UNIVERSITY OF ALBERTA

THE ROLE OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS IN VERY LOW DENSITY LIPOPROTEIN ASSEMBLY AND SECRETION

BY

DARREN GLENN FAST ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**.

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **THE ROLE OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS IN VERY LOW DENSITY LIPOPROTEIN ASSEMBLY AND SECRETION** submitted by **DARREN GLENN FAST** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

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This thesis is dedicated to my
wife and very best friend,
Linda Fast.
Thank you for everything!

Abstract

The aim of this study was to determine the role of phosphatidylcholine biosynthesis in the assembly and secretion of very low density lipoproteins (VLDLs). Young male rats, fed either a choline deficient (CD) diet or a control diet supplemented with 0.4% (w/w) choline (CS) for three days, were used as a model system. In this model system, VLDL secretion is impaired. No change in the amount of apoB in ER lumina was observed, but there was a 40-50% decrease of apoB in Golgi lumina from CD compared with CS rats. The defect in choline deficiency was shown not to be translocation of apoB into the microsomes, since treatment of microsomes from CD and CS rats showed similar protection on treatment with trypsin. When transport from the ER to the Golgi was blocked with brefeldin A, VLDL secretion was blocked, but no difference was observed in the degradation of apolipoprotein B (apoB) within the CD or CS cells. If increased catabolism of apoB were occurring in the endoplasmic reticulum of CD hepatocytes, enhanced degradation of apoB in CD cells might have been expected. Therefore, the degradation of apoB occurred in a post-ER compartment. The lipids of nascent VLDL from the lumina of ER and Golgi prepared from CD rat liver showed a relative enrichment of phosphatidylethanolamine, and depletion of phosphatidylcholine when compared to CS samples. Changes in nascent VLDL phospholipids mimicked the membrane phospholipid composition of organelles from which they were isolated. Analysis of the neutral lipids suggested that the CD particles contain more TG than corresponding CS particles, and this is supported by electron microscopy measurements. The phospholipid

composition of the organelles appears to be a factor in the final lipid composition of VLDL, and may affect the rate of bulk protein secretion from CD cells.

This study has shown that phosphatidylcholine biosynthesis is important in the assembly and secretion of very low density lipoproteins at an early stage in assembly, causing VLDL with altered phospholipid composition to be generated, and that nascent VLDL particles deficient in PC, are degraded in a post-ER compartment.

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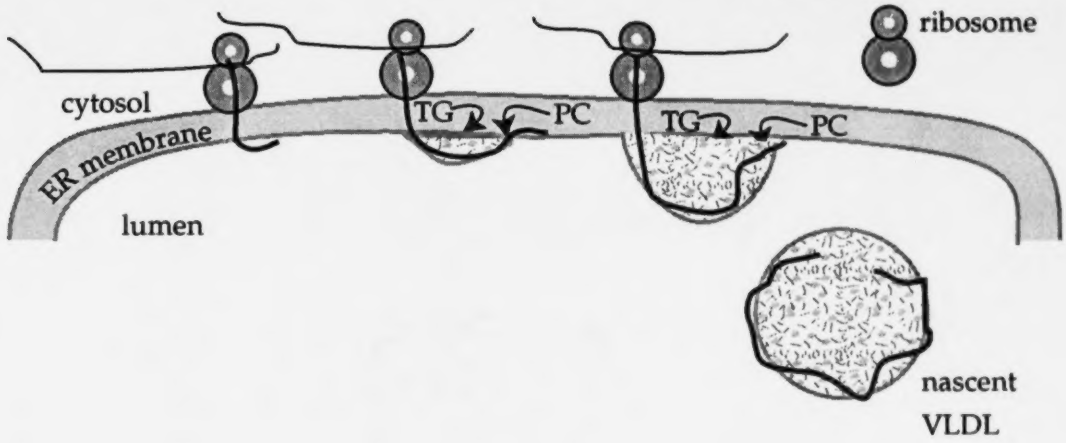
List of Abbreviations

ALLN	Acetyl-leucine-leucine-norleucinal
apoB	apolipoprotein B
apoE	apolipoprotein E
bestatin	[2S, 3R)-3-Amino-2-hydroxy-4-phenylbutanoyl]-L-leucine
BF	bottom fraction
BFA	Brefeldin A
BSA	bovine serum albumin
CD	choline deficient
CE	cholesteryl ester
CS	choline supplemented
CT	CTP: phosphocholine cytidylyltransferase
d	density
DMEM	Dulbecco's modified Eagle's medium
ELISA	enzyme linked immunosorbant assay
ER	endoplasmic reticulum
ER-I	heavy endoplasmic reticulum fraction
ER-II	light endoplasmic reticulum fraction
g	force of gravity
h	hour
HDL	high density lipoprotein
IDL	intermediate density lipoprotein
kDa	kilodaltons
LDL	low density lipoprotein

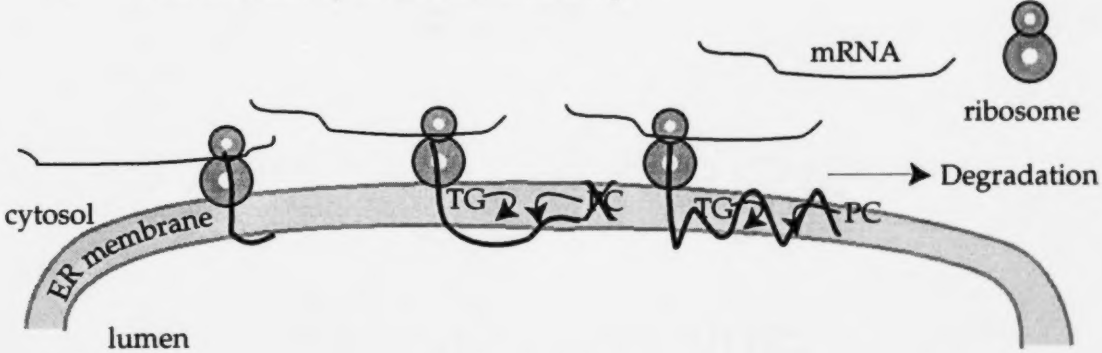
nm	nanometer
min	minute
MTP	microsomal triglyceride transfer protein
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEMT	PE methyltransferase
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene diflouride
S.D.	standard deviation
SDS	sodium dodecyl sulfate
S.E.M.	standard error from mean
TCA	trichloroacetic acid
TG	triacylglycerol
TLCK	1-chloro-3-tosylamido-7-amino-L-2-hepatanone
TPCK	L-1-tosylamido-2-phenylethyl choloromethyl ketone
VLDL	very low density lipoprotein

1 Introduction

The site of assembly of very low density lipoproteins is an area that has undergone much study over the last number of years. Elevated plasma LDL is the most frequently observed abnormality in patients with premature coronary artery disease (Avogaro *et al.*, 1979; Sniderman *et al.*, 1980). Atherosclerosis is responsible for up to 50% of all mortality in the industrialized world (Ross, 1993). In some cases elevated LDL levels are caused by impaired removal of LDL particles from the plasma (Langer *et al.*, 1972), but in the majority of cases, overproduction of apoB100, which is the main protein component of LDL, leads to elevated plasma LDL levels (Teng *et al.*, 1986; Venkatesan *et al.*, 1993; Grundy *et al.*, 1987). VLDL is a precursor of LDL and is synthesized in the liver of mammals. VLDL is converted to LDL by lipoprotein lipase mediated hydrolysis of the TG core, and removal of all apolipoproteins, except apoB (Teng *et al.*, 1986). Therefore, to understand the pathogenesis of increased plasma LDL caused by overproduction of apoB, it is crucial to understand the factors regulating hepatic secretion of VLDL. VLDL is assembled from apoB, TG, cholesterol, CE, and phospholipids (Sparks and Sparks, 1993; Borén *et al.*, 1993; Yao and McLeod, 1994). Reduced rates of TG, cholesterol, CE and phospholipid synthesis have been shown to inhibit VLDL assembly and target apoB to a degradation pathway (Sakata *et al.*, 1993; Yao and Vance, 1988; Dixon and Ginsberg, 1993; Adeli, 1994; White *et al.*, 1992; Borén *et al.*, 1993; Fungwe *et al.*, 1992; Sniderman and Cianflone, 1993; Tanaka *et al.*, 1993; Du *et al.*, 1994). Phospholipid and core lipids, as well as all of the apolipoproteins, are in a constant state of flux within plasma. Therefore, many studies have been aimed at one particular aspect of lipoprotein metabolism. Phospholipids form a monolayer on the surface of



A. Normal VLDL Assembly



B. VLDL Assembly in Choline Deficiency

Figure 1. Models of VLDL Assembly (1990).

Under normal conditions (A) apoB cotranslationally associates with neutral and phospholipid and buds from the ER membrane to form a nascent VLDL particle in the ER lumen. However, when PC biosynthesis was impaired, lipid addition to the particle was impaired (B), and apoB did not assemble with neutral lipid into a nascent VLDL particle, but rather was degraded by an undescribed intracellular pathway.

the particles that contains the hydrophobic neutral lipid core and allow movement of the neutral lipids through the aqueous environment of the blood. The ongoing biosynthesis of phosphatidylcholine, the major phospholipid of VLDL has been shown to be important in the secretion of VLDL (Yao and Vance, 1988; 1989; 1990). The present thesis is directed toward understanding the role of PC biosynthesis in the assembly and secretion of VLDL.

1.1 Initial Hypothesis of PC Biosynthesis and VLDL Assembly

At the time when this work was started, it was known that blocking PC biosynthesis inhibited VLDL secretion into hepatocyte medium (Yao and Vance, 1988) or rat plasma (Yao and Vance, 1990). The biosynthetic origin of PC was not an important factor since normal VLDL secretion could be restored by the addition of choline, methionine, which promotes PC biosynthesis via the methylation of PE, or lyso-PC, which can be acylated to PC in hepatocytes (Robinson *et al.*, 1989; Yao and Vance, 1990). Since the site of PC biosynthesis is the cytosolic face of the ER (Vance, 1990a), the initial hypothesis was that nascent lipoproteins would not be formed in choline deficiency as PC biosynthesis would be required for "budding" of the VLDL particle into the lumen of the ER. If particle formation was impaired, there should be fewer nascent VLDL particles in the lumina of the ER from CD, compared with CS, rat livers. In brief, the assembly of the particle was thought to be defective either as a result of a shortage of PC, or an inability of the particle to translocate across the ER membrane. The likely fate of excess apoB was thought to be degradation at the ER.

1.1.1 Model of Lipoprotein Assembly (1990)

The working model of PC biosynthesis in VLDL assembly and secretion as it was understood at the start of this project is shown in Figure 1.

Initially, apoB mRNA bound to ribosomes would attach to the ER membrane. The signal sequence of apoB would be cleaved and the remainder of apoB would translocate across the ER membrane and co-translationally associate with lipid. If PC biosynthesis was inhibited (i.e., choline deficiency), lipids would not be added to apoB, and apoB would remain associated with the ER membrane and be targeted for degradation by an unknown mechanism. However, if PC biosynthesis were ongoing, lipid would be added to apoB and a nascent VLDL particle would bud from the membrane into the lumen of the ER where it would then rapidly move into the Golgi. An alternate scheme involved the apoB moving from the ER to the Golgi while bound to the membrane and associating with a lipid droplet to form a nascent VLDL particle (Alexander *et al.*, 1976). In the Golgi, the apoB moiety of the nascent VLDL is modified by post-translational modifications such as processing of N-linked carbohydrates, acylation and phosphorylation (Yao and McLeod, 1994; Davis, 1993; Dixon and Ginsberg, 1993; Chan, 1992). After exiting the Golgi, the particles would be transported to the plasma membrane by secretory vesicles where the VLDL would be released into the space of Disse by exocytosis.

1.2 General Approach and Objectives

PC is the major phospholipid component of VLDL comprising approximately 60% of the total phospholipid. It is located exclusively on the surface of the particle. We have been interested in the role of PC biosynthesis in the assembly and secretion of VLDLs from rat hepatocytes. Our approach has been to inhibit selectively PC biosynthesis by feeding 50 g rats a CD diet for 3 days. This causes a 60% reduction of VLDL in the plasma and a corresponding accumulation (6.5 fold) of TG in the liver (Yao and Vance, 1988). The primary objective of this study was to define the interactions of phosphatidylcholine and apoB in the formation of VLDL. The methods we used to achieve our objective included determination of the subcellular localization of the defect of VLDL secretion and/or assembly in choline deficiency, and to determine the fate of non-secreted apoB under these conditions. I also examined the nascent VLDL particles in both CD and CS states to determine potential mechanisms for removal of CD particles. This was accomplished by examining the density distribution and lipid composition of nascent lipoprotein particles from the lumina of the secretory pathway. Localization of the degradation site was accomplished with intracellular transport inhibitors.

1.3 Phosphatidylcholine

PC, commonly called lecithin, is an important component of cell membranes, lipoproteins, and surfactant (Vance and Vance, 1990a; 1990b; Vance, 1990a). Phosphatidylcholine accounts for up to 70% of the phospholipid of mammalian cells. The breakdown products of agonist induced phospholipase-A_2 catabolism of PC are involved in signal transduction (reviewed by Exton, 1990; 1994). The major role for PC in lipoproteins is believed to be as a surface component where it is exclusively located. This coat is essential to contain the large hydrophobic neutral lipid core. Phosphatidylcholine is thought to be added to the nascent VLDL in the ER membrane where the enzymes for its biosynthesis are found (Vance and Vance, 1990a; 1990b). Phosphatidylcholine is thought to be essential for mammalian life as no known hereditary diseases of its biosynthetic pathways are known (Tronchère *et al.*, 1994).

1.3.1 Choline

Choline is the headgroup of PC and is requried for PC biosynthesis. Choline was first identified more than a century ago, and its apparent deficiency syndrome was accidentally discovered by Best and Huntsman (1932) while studying the effect of insulin on blood sugar in the depancreatized dog. These studies were the first to recognize PC and choline as the active ingredients in the pancreas. Choline was recognized as a lipotropic factor as animals fed a diet devoid of choline developed fatty livers (Best and Huntsman, 1932). A lipotrope is defined as a substance which "decreased the rate of deposition or accelerated the removal of liver fat" (Olson, 1958).

Choline is a required component in the biosynthesis of PC (Figure 2), lyso-PC, choline plasmalogen, and sphingomyelin, and is ubiquitous in mammalian tissues. Choline is also a precursor of the neurotransmitter acetylcholine, and is an important donor of methyl groups (Zeisel, 1994). Although choline can be synthesized endogenously in some species, it is required in the diet of rats, hamsters, guinea pigs, dogs, cats, and pigs (Zeisel, 1990; Zeisel *et al.*, 1991). Choline is taken up into cells by a combination of diffusion and mediated transport (Zeisel, 1994). Essentially, there are three fates of dietary choline as shown in Figure 2 (reviewed in Zeisel, 1994): 1. a small fraction is acetylated to acetylcholine by choline acetyltransferase in cholingeric neurons, 2. oxidation to betaine by the action of choline dehydrogenase and betaine aldehyde dehydrogenase or, 3. phosphorylation to phosphocholine by choline kinase, the first step in the major pathway of PC biosynthesis (Vance, 1990a). Under normal conditions in rat liver much greater amounts of choline are oxidized to betaine (9 μmol/h/g) than phosphorylated to phosphocholine (1 μmol/h/g). However, when choline amounts are limiting (i.e., choline deficiency) essentially all choline is phosphorylated to phosphocholine (Pomfret *et al.*, 1990). The major non-dietary source of choline is the *de novo* biosynthesis of PC via the methylation of PE, by PEMT, and the subsequent catabolism of PC to choline. This enzyme sequentially methylates PE using *S*-adenosyl-L-methionine as a methyl donor (Bremer and Greenberg, 1961; Ridgway, 1989). Most PEMT activity is found in liver with significant activity found in brain and mammary tissue. An alternative pathway for choline biosynthesis exists in brain, where phosphocholine is formed by the sequential methylation of phosphoethanolamine (Andriamampandry *et al.*, 1992). However, this activity has not been demonstrated in rat liver. The major pathways for choline metabolism are diagramatically outlined in Figure 2, and it can be seen that choline and methionine metabolism are interrelated and converge at the regeneration of methionine from homocysteine.

1.3.2 Biosynthesis

There are three main pathways for the biosynthesis of PC (Figure 2). The major pathway for PC synthesis was first described by Kennedy and coworkers in the 1950's (Kennedy and Weiss, 1956). This pathway is termed the CDP-Choline pathway and has been estimated to account for 60-80% of PC biosynthesis under normal conditions (Sundler and Åkesson, 1975). The rate limiting step in this pathway is the enzyme CTP: phosphocholine cytidylyltransferase, a 42 kDa protein that has two forms (Vance, 1989; 1990a).

Biosynthesis of PC also occurs via sequential methylation of PE (Bremer and Greenberg, 1961). This involves three sequential methyl group transfers from *S*-adenosyl-L-methionine to PE catalyzed by PEMT (Ridgway, 1989). This pathway may account for 20 to 40% of the hepatic PC synthesis. Phosphatidylcholine can also be formed by the reacylation of lyso-PC with acyl-CoA (Lands, 1960).

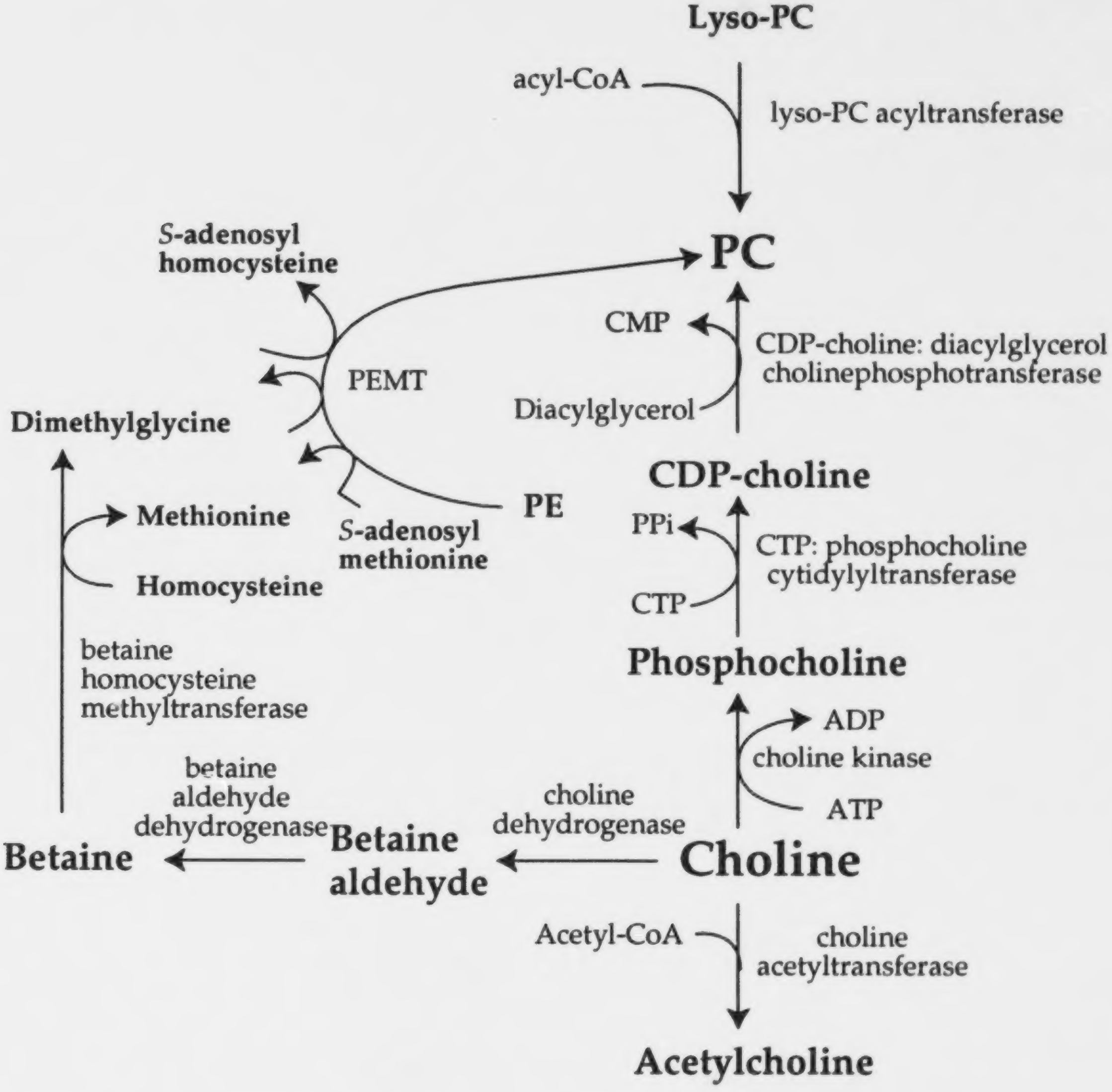


Figure 2. **Choline Metabolism and Phosphatidylcholine Biosynthesis**
The major uses for choline are as a precursor of PC biosynthesis, as a methyl donor, and as a precursor of acetylcholine. This diagram also shows the three pathways of PC biosynthesis, from choline via the CDP-choline pathway, via the sequential methylation of PE and via the acylation of lyso-PC.

1.3.3 Regulation of Phosphatidylcholine biosynthesis

The synthesis of PC via the CDP-choline pathway is regulated by the enzyme CTP: phosphocholine cytidylyltransferase (reviewed in Vance, 1990a). This enzyme catalyzes the formation of CDP-choline from CTP and phosphocholine. The main mechanism for CT regulation is translocation of the enzyme from a cytosolic pool, where it is inactive, to the ER membrane where it is active (reviewed in Vance, 1990a; Tronchère *et al.*, 1994). Factors that reduce the activity of CT include high PC levels, low diglyceride or fatty acid levels, and phosphorylation of the enzyme. Factors that promote translocation of CT to the membrane include; low PC levels, increased diglycerides or fatty acids and dephosphorylation of the enzyme. CT may also be regulated by channeling of choline precursors, aggregation of CT, and at the level of gene expression (reviewed by Tronchère *et al.*, 1994). In choline deficiency CT is mainly located at the ER membrane, but synthesis of PC is blocked due to a lack of phosphocholine (Yao *et al.*, 1990; Jamil *et al.*, 1990).

1.3.4 Role of Phosphatidylcholine in Plasma Lipoproteins

Phosphatidylcholine has several roles in plasma lipoproteins. The primary role of PC is structural, providing a barrier around the hydrophobic neutral core of the particles, protecting the core from the aqueous environment of the plasma. PC is also a donor of fatty acid groups for synthesis of CE, by the action of lecithin cholesterol acyltransferase (reviewed in Jonas, 1991). VLDL secretion is also dependent on PC (Yao and Vance, 1988; Yao and Vance, 1990). Direct studies on the role of PC biosynthesis on VLDL assembly and secretion are the main theme of this thesis.

1.4 Choline Deficiency Model

Choline deficiency is defined as a diet devoid of choline, but with adequate, but not excessive levels of methionine plus folic acid and vitamin B_{12} (Ghoshal and Farber, 1993). The diet that I have used in these studies meets these requirements. Rats fed a CD diet experience normal weight gains, but with rapid development of fatty liver. There is early necrosis (4.5 to 5 days) of the liver affecting at least 50% of the hepatocytes by 2 weeks. This is thought to be caused by lipid peroxidation and aldehyde production in hepatocytes. Hence, there is rapid turnover of hepatocytes from rats fed a CD diet (Zeisel, 1994). Fatty cysts and cirrhosis of the liver are rarely seen. However, after 2 years of CD diet, 50 to 70% of male rats will develop hepatocellular carcinomas compared to less than 5% of controls (Ghoshal and Farber, 1993). Choline is the only single nutrient for which dietary deficiency causes hepatocarcinoma without known carcinogens (Lombardi *et al.*, 1991; Zeisel, 1993; Zeisel and Blusztajn, 1994) although this requires extended periods of time on a CD diet and therefore is not directly relevant to this study.

Lipotrope deficiency is defined as devoid of choline, methionine, betaine, folic acid and/or vitamin B_{12}. This diet has not been rigourously defined and therefore the effects due to lipotrope deficiency have been varied and often confused with pure choline deficiency (Ghoshal and Farber, 1993). This is compounded by the fact that choline, methionine and folate metabolism are interrelated (Figure 2) and that depleting any one of these will have an effect on the metabolism of the other two. An animal fed a lipotrope deficient diet has several major pathological differences from one fed a CD diet. The lipotrope deficient animal will have poor weight gain, but with rapid development of fatty liver (however in the central zone rather than the periportal zone seen in choline deficiency). After several weeks fatty cysts are seen, and cirrhosis of the liver is very common. The development of hepatocellular carcinomas is uncertain in lipotrope deficient animals (Ghoshal and Farber, 1993).

The earliest and most obvious manifestation of choline deficiency is the rapid development of fatty liver and reduction of hepatic PC/PE ratios (Malhotra and Kritchevsky, 1978; Windmueller, 1964; Kapoor *et al.*, 1992). The increased intracellular TG is thought to be as a result of impaired lipoprotein secretion (Lombardi, 1971; Yao and Vance, 1988) and it is found in the cytosol. Alternatively, the increased TG may be due to an accumulation of PC precursors such as diacylglycerol that would be converted into TG. Levels of TG in microsomal membranes do not appear to change in CD compared to CS states (Lombardi *et al.*, 1968). Choline deficiency selectively inhibits PC biosynthesis by 70%, and this leads to a selective inhibition of VLDL secretion (Yao and Vance, 1988).

1.4.1 Physiological Effects of Choline Deficiency

In animals, choline deficiency has been associated with fatty liver, growth impairment, infertility, renal lesions and memory loss (reviewed in Zeisel, 1990). It has been observed that young rats have a greater demand for choline than do old rats (Lombardi, 1971).

Adult human males fed a semisynthetic diet deficient in choline for 6 weeks did show signs of liver damage (release of alanine aminotransferase into serum) and had lower plasma choline and PC levels (Zeisel *et al.*, 1991). However, the requirement for choline in the diet of humans has not been determined as most foods contain choline and/or PC. Choline deficiency may occur in humans under unusual circumstances, such as malnutrition or cirrhosis, which may cause fatty livers. This also occurs in some patients after intestinal bypass surgery and in some receiving intensive nutrition support (Zeisel, and Blusztajn, 1994).

CD does not affect protein synthesis, but does slow the rate of release of newly synthesized proteins into plasma (Lombardi and Oler, 1967). Choline deficiency also causes fragmentation and dilation of ER and Golgi membranes (Estes and Lombardi, 1969). The implication for the rate of protein secretion is unclear.

1.4.1.1 Triacylglycerol Metabolism

Triacylglycerol used for lipoprotein assembly is synthesized by a three-step process from glycerol 3-phosphate. First, the glycerol 3-phosphate is converted to phosphatidic acid by the action of 2 specific acyltransferases, this is then converted to diacylglycerol via phosphatidic acid phosphohydrolase, finally, TG is synthesized from diacylglycerol, at the cytosolic face of the ER, via diacylglycerol acyltransferase (Brindley, 1991). These enzymes are present in or on the ER and other subcellular organelles. This pathway is stimulated by oleic and palmitic acids. TG synthesis is not affected by choline deficiency (Lombardi, 1971; Yao and Vance, 1988), however there is increased intracellular accumulation in the cytosol, but not in the microsomal membranes (Lombardi *et al.*, 1968). The failure to secrete VLDL observed in animals fed a CD diet likely leads to deposition of TG in the cytosolic pool (Norum *et al.*, 1983). Pulse-labeling studies with oleic acid suggest that newly synthesized, rather than existing TG, is preferentially used for secretion in VLDL (Ide and Ontko, 1981) In the absence of extracellular fatty acids it has been shown that TG used for VLDL secretion in primary rat hepatocytes was mainly derived (70%) from the lipolysis of cytosolic TG and subsequent re-esterification in or at the ER (Wiggins and Gibbons, 1992; Gibbons, 1994).

1.4.1.2 Phospholipid Metabolism

PC biosynthesis is inhibited by 70% in CD hepatocytes (Yao and Vance, 1988), with rapid change in phospholipid compositions of membranes (Lomardi, 1971; Pomfret *et al.*, 1990). In choline deficiency, there is a decrease in the PC/PE ratio of microsomes, this is caused by a decrease of PC and an increase of PE (Kapoor *et al.*, 1992). The source of the PE is either blocked methylation or an increase in PE precursors, as a result of decreased PC biosynthesis. Vance and Vance (1986) showed that phospholipids used in the assembly of lipoproteins were not from a uniformly labeled pool. Rather, there was a preferential secretion of PC and PE labeled from serine, but not from ethanolamine. Phospholipids derived from choline precursors were labeled and secreted with approximately the same specific activity. Thus, there is not a rapid equilibrium of phospholipids derived from different biosynthetic origins.

Blocking the methylation pathway of PC biosynthesis with 3-deazaadenosine does not interfere with VLDL secretion (Vance *et al.*, 1986), suggesting that the methylation pathway of PC biosynthesis is not required for VLDL secretion. It has also been shown that sphingolipids are secreted on VLDL particles and that choline deficiency does not affect sphingolipid synthesis (Merrill, A.H. Jr., Lingrell, S., Wang, E., Nikolova-Karakashian, M., and Vance, D.E., unpublished data).

1.4.2 Effects of Choline Deficiency on Bulk Protein Secretion

In CD rats, there appears to be a decrease in the rate intracellular transport of albumin, resulting in a delay in the secretion. The change in albumin secretion rate appears transient as steady state levels of albumin in plasma are equivalent between CD and CS rats (Lombardi and Oler, 1967; Oler and Lombardi, 1970). The steady state levels of VLDL from CD plasma are decreased relative to CS plasma, in the same experimental protocol. The change in secretory rate, caused by choline deficiency, may be due to changes in the ultrastructure of the subcellular organelles (Estes and Lombardi, 1969).

1.4.3 Effects of Choline Deficiency on Lipoprotein Secretion

One of the earliest effects of choline deficiency is an interference in the metabolism of hepatic lipoprotein secretion (Lombardi, 1971). This effect has been observed after as little as 8 h on a CD diet. Choline deficiency has been shown to block specifically VLDL, but not HDL secretion (Yao and Vance, 1990). The effect is also rapidly reversed by the addition of choline or other substances that can promote the synthesis of PC, such as methionine, betaine, homocysteine or lyso-PC (Yao and Vance, 1988; Robinson *et al.*, 1989). It is not reversed by closely related headgroups such as ethanolamine, N-monomethylethanolamine or N,N-dimethylethanolamine (Yao and Vance, 1990).

1.4.3.1 Site of Phosphatidylcholine Incorporation into Lipoproteins

The site of PC addition to VLDL is thought to be at the ER membrane as the particle is formed (Vance and Vance, 1990a). Although the Golgi has the capacity to synthesize PC, PE and PS, which may be added to nascent VLDL particles (Vance and Vance, 1988); nascent VLDL particles with the same lipid composition as plasma VLDL can be observed in the ER (Rusiñol *et al.*, 1993a). Studies with labelled precursors of phospholipids suggest that phospholipids and their precursors are compartmentalized into defined intracellular pools and are used for different purposes (Graham *et al.*, 1988; Vance, and Vance, 1986; 1990a; 1990b). Studies also show that the fatty acid composition of PC in plasma is different from liver (Vance and Vance, 1985). Lipoproteins secreted from primary hepatocytes also have different fatty acid composition than do the hepatocytes themselves (Vance and Vance, 1986).

1.4.3.2 Pools of Phosphatidylcholine precursors

In addition to compartmentalization of phospholipids, there may also be compartmentalization of precursor molecules. For example, diacylglycerol is a precursor of PC, and is also used as a second messenger in cells, therefore, the levels of diacylglycerol must be tightly regulated (Zeisel, 1993). The aqueous precursors of phospholipid synthesis are also thought to reside in pools. Chanelling of aqueous intermediates of PC biosynthesis has been observed in cultured glioma cells (George *et al.*, 1989; 1991). This suggests that the enzymes involved in the biosynthesis of PC may be arranged in an organized fashion by membranes and cytoskeleton (Vance and Vance, 1990a), and may have implications for the assembly of PC into nascent VLDL particles.

1.4.4 Relevance of Choline Deficiency Model

The CD model as a system for study of lipoprotein assembly has a number of advantages.. It is easy to prepare subcellular fractions and examine the steady state levels and composition of nascent lipoproteins. Also, the CD effect is relatively rapid, occuring maximally after 3 days of feeding. It is possible to use whole animal studies, or hepatocyte preparations that allow for easy labelling and inhibitor studies. The CD effect on VLDL secretion is very rapidly reversed, with normal VLDL secretion restored after 4 h of supplementation with choline (Yao and Vance, 1988).

Some disadvantages of using a rat model include the inability to distinguish between VLDL particles containing apoB100 or apoB48, since both apoB100 and apoB48 are assembled into VLDL sized particles. Also, it is not feasible to stably transfect the primary hepatocytes used in this thesis with other proteins (i.e., truncated forms of apoB) to study the effect of choline deficiency on portions of the apoB molecule. Also, rat lipoprotein metabolism is distinct from that of humans (Suckling and Jackson, 1993; Gibbons, 1994).

1.5 Lipoprotein Classes

Plasma lipoproteins are aggregates of specific lipids and proteins. Their role is to transport water insoluble compounds (mainly TG and CE) from their site of synthesis (liver or intestine), or point of absorption to peripheral tissues. The amphipathic coat (a monolayer of phospholipids and cholesterol) permits the transport of the hydrophobic core lipids through the aqueous mileu of the blood, to other tissues, while allowing access of lipases to the core lipids. Approximately 80% of lipoproteins originate from the liver (Wu and Windmueller, 1979). There are several classes of lipoproteins most commonly defined according to their buoyant density (Table I). The classes of lipoproteins have distinctly different protein, phospholipid and neutral lipid compositions as shown in Table II. Within any given class of lipoproteins, there is considerable heterogeneity of lipid and apolipoprotein composition (Davis, 1991). The density and exact lipid composition of apoB containing lipoproteins is dependent on the nutritional state of the animal or cultured cells (Dixon and Ginsberg, 1993; Abate *et al.*, 1993). Most of the apolipoproteins (E, C-I, C-II, C-III, A-I, A-II, and A-IV) are exchangeable between lipoprotein particles. ApoB however, is a non-exchangeable apolipoprotein. In plasma, lipoproteins are in dynamic equilibrium with each other and with various tissues and membranes within the body (reviewed in Fielding and Fielding, 1991). Many model systems have been used to study lipid and lipoprotein metabolism, and the merits and shortcomings of each have recently been reviewed (Gibbons, 1994, Suckling and Jackson, 1993).

Table I

Physical Properties of Plasma Lipoproteins

Lipoprotein Class	Diameter (nm)	Molecular Weight (kDa)	Buoyant Density (g/ml)
Chylomicrons	75 - 1200	400,000	0.93
VLDL	30 - 80	10-20,000	0.93 - 1.006
IDL	25 - 35	5 - 10,000	1.006 - 1.019
LDL	18-25	2,300	1.019 - 1.063
HDL2	9-12	360	1.063 - 1.125
HDL3	5-9	175	1.125 - 1.21

From Smith *et al.*, 1983

Table II

Composition of Rat Lipoproteins

The percent composition by weight of various components of rat plasma lipoproteins

Lipoprotein Class	Proteins	Phospholipids	Cholesterol	Neutral Lipids
VLDL	9.4	12.6	2.7	75.4
LDL	24.9	21.2	8.6	45.3
HDL	41.4	26.0	14.8	17.8

from Mjos *et al.*, 1975 and Pasquali-Ronchetti *et al.*, 1975

1.5.1 Chylomicrons

Chylomicrons are produced solely in the intestine (Yao and McLeod, 1994), and secreted into the lymph. These are very large particles (diameter 75-1200 nm) containing apoB48, apoA-IV and apoA-I. TG is the major core lipid of chylomicrons giving these particles a very low buoyant density (0.93 g/ml). The size and lipid composition of chylomicrons is affected by diet, and production is subject to hormonal regulation (reviewed by Young, 1990). The catabolism of chylomicrons is very rapid with a half life of 5-10 min in plasma (Young, 1990). Chylomicrons are converted to chylomicron remnants that have lost apolipoprotein As and gained apoE. The remnant particles are smaller having had much of their TG core hydrolyzed by lipases. The remnant particles are cleared by the LDL receptor in the liver (reviewed by Young, 1990).

1.5.2 Very Low Density Lipoproteins

Very low density lipoproteins are produced by the liver and are the precursors of intermediate density lipoproteins and LDL. They have a density of 0.93 - 1.006 g/ml and diameters of 30-80 nm. The particles are largely composed of TG with some CE and are surrounded by one molecule of apoB and a shell of phospholipid with some cholesterol. VLDL particles are rapidly converted to IDL and LDL mainly by the action of lipoprotein lipase an enzyme associated with endothelial cell surfaces (see section 1.6.5). VLDL will be discussed in greater detail in section 1.6.

1.5.3 Intermediate Density Lipoproteins

These particles are mainly produced by the catabolism of VLDL by lipases. They are classified as having a density range of 1.006-1.02 g/ml. These particles may undergo further catabolism to LDL, or may be cleared directly by the liver via the LDL receptor (Schneider, 1991).

1.5.4 Low Density Lipoproteins

These lipoproteins are classified as having a buoyant density of 1.02-1.063 g/ml. They are produced in plasma by the lipolysis of VLDL and IDL. The clearance of LDL from plasma has recently been reviewed (Schneider, 1991). In humans, apoB100 is the sole apolipoprotein associated with this class of lipoproteins. High plasma LDL levels are used clinically as a marker of atherosclerosis risk.

1.5.5 High Density Lipoproteins

High-density lipoproteins (HDLs) are a heterogeneous group of particles defined either by size or by apolipoprotein content. HDL has a buoyant density in the range of 1.063-1.21 g/ml and contains one or more of the following apolipoproteins (A-I, A-II, A-IV, C-II, C-III or E). There are several subfractions of HDL that appear to have distinct, but interrelated metabolic functions, including facilitation of CE transfer to LDL and VLDL, modulation of TG-rich particle catabolism, and possibly, removal of cholesterol from peripheral tissues. HDL subfractions also are markers for epidemiological risk for coronary artery disease. Because they provide information about the physiological processes of cholesterol metabolism, HDL subfractions are an important tool in the study of the relationship between lipids and cardiovascular disease (reviewed by Silverman *et al.*, 1993; Miller, 1990; Tall, 1992; Catapano *et al.*, 1993).

1.6 Very Low Density Lipoproteins

A VLDL particle is an aggregate consisting of a neutral lipid core surrounded by a shell of polar lipids and specific apolipoproteins. Very low density lipoproteins are synthesized mainly in liver and are classified by having a buoyant density of 0.93-1.006 g/ml. The principal apolipoproteins of rat VLDL are apoB (either apoB100 or apoB48) and apoE with small amounts of apoC (reviewed by Dashti, 1991). The main component of the core is TG with very little CE (reviewed by Dashti, 1991).

1.6.1 Role of VLDL

The main role of VLDL is to carry TG as an energy source for peripheral tissues. The TG may be mobilized from adipose tissue under certain conditions. Triacylglycerol is a very concentrated source of energy, providing six times more energy per gram than hydrated glycogen (Gibbons and Angelin, 1994). In the starved rat, TG from VLDL provides 50% of lipid energy requirement of muscle, suggesting that VLDL is a major player in energy metabolism. However, TG is very hydrophobic and cannot traverse the aqueous environment of the plasma. Therefore, TG is combined with apoB and phospholipid to generate a water soluble complex (VLDL), that can easily be transported through the plasma, and deliver the energy to the peripheral tissues. Once at the tissue, TG is hydrolyzed by lipases, the fatty acids enter the cell and are utilized via β-oxidation (reviewed in Schulz, 1991a; 1991b; Guzmán and Geelen, 1993).

The concentration of lipoproteins in the serum is balanced by the relative rates of production and catabolism. Since high levels of apoB in plasma are an indication for premature coronary atherosclerosis, much study has focused on the clearance of VLDL and LDL from plasma (reviewed in Fielding, 1992; Schneider, 1991). Much study has also focused on the regulation of assembly and secretion of very low density lipoproteins (reviewed in; Yao and McLeod, 1994; Sparks and Sparks, 1993; Dixon and Ginsberg, 1993; Sniderman and Cianflone, 1993; Borén *et al.*, 1993) as a mechanism for regulating plasma apoB levels. The main focus presented in this thesis will deal with the regulation of VLDL assembly and secretion as affected by PC biosynthesis.

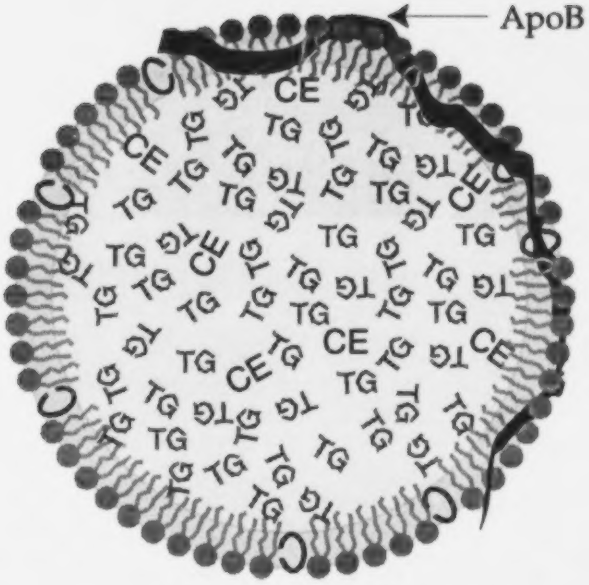


Figure 3. Cross Sectional View of a VLDL Particle
VLDL is comprised of a polar lipid mononlayer composed of phospholipid and cholesterol (C). ApoB is integrated into the surface layer. Other apolipoproteins are present on the surface, but are not shown for clarity. The core consists mainly of triacyl-glycerols (TG) with some cholesteryl ester

1.6.2 Structure of VLDL

VLDL consists of a core of hydrophobic lipids (mainly TG with some CE), surrounded by a monolayer of phospholipids, cholesterol and a single molecule of either apoB100 or apoB48, and a few other proteins such as apolipoprotein-E and apolipoprotein C (Gibbons, 1990; Young, 1990; Dixon and Ginsberg, 1993). VLDLs have a diameter of 30-80 nm, and are considered to be globular in nature (Gibbons, 1990). A cross-sectional view of a VLDL particle is shown in Figure 3.

1.6.2.1 ApoB

ApoB is an essential structural component required for the assembly and secretion of VLDL, and is a non-exchangeable protein. There is one molecule of apoB per VLDL (Elovson *et al.*, 1988), therefore the amount of VLDL secreted is directly related to apoB secretion. There are two forms of apoB; apoB100, a full length form, and apoB48, the amino-terminal 48% of apoB100. ApoB contains both hydrophobic and hydrophilic sequences (reviewed in Chan, 1992). ApoB interaction with arterial tissue via the LDL receptor has made it a subject of clinical interest (Olofsson *et al.*, 1987a).

1.6.2.1.1 ApoB Synthesis

ApoB shows tissue specific expression and is mainly synthesized in liver and intestine (Yao and McLeod, 1994). The human gene spans 43 kilobases and contains 29 exons and 28 introns. In most species there are two forms of apoB; apoB100 and apoB48. ApoB48 is the amino-terminal 48% of apoB100. Both forms of apoB are made from a single gene. ApoB48 mRNA is a post-transcriptional modification of full length apoB mRNA. The process that occurs is the deamination of cytidine 6666 to form a uridine (Shah *et al.*, 1991; Hodges and Scott, 1992) by an mRNA editing enzyme (Bhattacharya *et al.*, 1994; Teng *et al.*, 1993). The editing activity has been localized to the nucleus (Lau *et al.*, 1991). The net result is conversion of a Gln codon (CAA) to a termination codon (UAA). The editing mechanism is tissue specific and is developmentally and metabolically regulated (reviewed in Yao and McLeod, 1994). In humans, the liver produces only apoB100 while the intestine produces apoB48. However, rat liver produces both apoB100 and apoB48 (Wu and Windmueller, 1981; Kane, 1983), and rat intestine produces mainly apoB48.

ApoB, is constituitively synthesized in excess (Yao and McLeod, 1994), and apoB secretion is post-translationally regulated. The time for apoB100 translation in the liver has been shown to be 10-14 min (Boström *et al.*, 1986; Olofsson *et al.*, 1987b). The time for secretion is an additional 30 min. ApoB contains a cleavable signal sequence that directs apoB to the ER membrane. Newly synthesized apoB cotranslationally associates with the ER membrane (Boström *et al.*, 1986; 1988; Borén *et al.*, 1990), although it appears that not all the apoB is fully translocated into the ER lumen (Davis *et al.*, 1990; Du *et al.*, 1994; Boström *et al.*, 1986; Borén *et al.*, 1992; Dixon *et al.*, 1992; Bamberger and Lane, 1988; Davis *et al.*, 1989; Wilkinson *et al.*, 1992b) as a significant amount is exposed on the cytoplasmic face of the ER, and can be digested with exogenous proteases (Davis *et al.*, 1990; Dixon *et al.*, 1992). The membrane bound apoB is glycosylated (Thrift *et al.*, 1992; Borén *et al.*, 1990), and the signal sequence cleaved (Borén *et al.*, 1990), suggesting that apoB spans the membrane at least once. ApoB is not expected to integrate into the membrane as apoB does not contain traditional transmembrane sequences (Knott *et al.*, 1986; Yang *et al.*, 1986). The membrane bound apoB has a higher degree of glycosylation than plasma apoB (Wong and Torbati, 1994). It may be that apoB requires cofactors, or undergoes a novel translocation process (Lingappa, 1991; Chuck and Lingappa, 1992) to span the membrane as it is being synthesized. As apoB is being translated, it may undergo one of at least two fates. First, it may translocate to the ER lumen where it would join with neutral and phospholipids to form a nascent VLDL particle, which would then be secreted, or secondly remain associated with the ER membrane, and be degraded (Thrift *et al.*, 1992; Furukawa *et al.*, 1992; Borén *et al.*, 1992; Du *et al.*, 1994). The synthesis and secretion of apoB containing lipoproteins have recently been reviewed (Davis, 1993; Dixon and Ginsberg, 1993, Yao and McLeod, 1994, Borén *et al*, 1993; Sniderman and Cianflone, 1993; Sparks and Sparks, 1993).

1.6.2.1.2 ApoB Structure

The primary structure of apoB is known (Cladaras *et al.*, 1986; Knott *et al.*, 1986, Yang *et al.*, 1986). ApoB100 is one of the largest proteins known, containing 4536 amino acids (human). The structure of apoB has been reviewed (Olofsson *et al.*, 1987b; Young, 1990; Chan, 1992). ApoB48 (human) contains the amino-terminal 2152 amino acids of apoB100 and is produced by a post-transcriptional modification of apoB100 mRNA. ApoB has two regions of homology to the sequence of apoE thought to be important for LDL-receptor binding (Chan, 1992). This would allow apoB to be recognized by the LDL receptor. The primary sequence of apoB contains 19 potential N-glycosylation sites of which 16 are used (Chan, 1992). Up to 37% of the N-linked oligosaccharides contain high mannose structures, which is unusually high for a secretory protein (Olofsson *et al.*, 1987b). ApoB has also been shown to be phosphorylated on serine residues (Davis *et al.*, 1984; Jackson *et al.*, 1990; Sparks *et al.*, 1988; Sparks and Sparks, 1990a). The 25 cysteine residues of apoB100 are asymmetrically distributed with a concentration in the amino terminal 15% of the molecule. Sixteen of the cysteines are involved in disulfide links (Chan, 1992). It is possible that this amino-terminal region forms a globular structure (Olofsson *et al.*, 1987b; Borén *et al.*, 1991). The secondary structural predictions of apoB suggest that it contains 43% α-helix, 21% β-sheet, 20% random coil and 16% β-turns (Chan, 1992). Chatterton *et al.* (1991) have shown by immunoelectron microscopy that apoB spans at least a hemisphere of human LDL. Analysis of the amino acid sequence and tryptic peptides have indicated numerous hydrophobic regions are present in apoB, and they are widely distributed throughout the primary sequence (Chan, 1992). However none contain a typical transmembrane sequence, nor are any of the hydrophobic regions greater than 13 amino acids (Chan, 1992; Dixon and Ginsberg, 1993). ApoB is thought to interact with the hydrophobic environment of lipoproteins via short (approximately 5 amino acids) amphipathic β-strands. These amphipathic β-strands have a consensus sequence of acidic-aromatic-polar-aliphatic-proline and are clustered in four proline-rich domains, located between amino acids 1167 and 3865 (Knott *et al.*, 1986; Olofsson *et al.*, 1987b; Scott *et al.*, 1987). The two types of peptide sequences thought to be involved in lipid binding include amphipathic α-helices and proline rich hydrophobic sequences with β-sheet potential (Chan, 1992). It appears that a minimum amount of apoB sequence is required to bind lipids. Studies using carboxy-truncated apoBs expressed in either HepG2, or rat hepatoma cells suggest that apoB18 and apoB23 are not associated with core lipids (i.e., they did not have a density of < 1.21 g/ml), but constructs expressing apoB28 or longer were associated with lipid, formed lipoprotein particles and exhibited a progressive decrease in density with increasing length (Graham *et al.*, 1991; McLeod *et al.*, 1994; Yao *et al.*, 1991; Spring *et al.*, 1992). The increasing length of the apoB construct expressed correlated with the core circumference in a linear manner (reviewed in Yao and McLeod, 1994). This suggests that information directing the assembly of apoB containing lipoproteins is found over the entire length of the apoB molecule. The only exception to this is the N-terminal 15-17% of the molecule, which contains a cysteine rich region believed to form a disulfide stabilized globular structure (Borén *et al*, 1993). It is also noted that apoB undergoes fatty acylation with either stearic or palmitic acid (Hoeg *et al.*, 1988). Acylation may greatly increase the hydrophobicity of apoB and its ability to associate with lipids (Olofsson *et al.*, 1987a).

ApoB contains an amino terminal signal sequence that should target it for translocation across the ER membrane (Protter *et al.*, 1986). This signal sequence is cleaved in HepG2 cells (Borén *et al.*, 1993), and Pease *et al.* (1991) have shown that the amino terminus of apoB is cotranslationally inserted into the inner leaflet of the ER. ApoB does contain novel pause transfer sequences (Chuck and Lingappa, 1992). These are "33 amino-acid sequences" containing charged amino acids that appear to transiently pause the translocation of apoB *in vitro*. ApoB does not contain typical stop transfer sequences as these would target apoB to become a membrane protein (Borén *et al.*, 1991) or any specific domains that are responsible for membrane integration (Shelness *et al.*, 1994). Some apoB appears to be membrane-bound (Borén *et al.*, 1990; Davis *et al.*, 1990) and this is thought to be a pool of apoB that is not used for lipoprotein assembly and therefore targeted to a degradation pathway. ApoB is extremely hydrophobic, as it is completely insoluble in aqueous solutions after delipidation, and therefore must remain associated with lipids.

ApoB in humans can be associated with apolipoprotein(a), a large protein that is structurally related to plasminogen, via a disulfide link (Phillips *et al.*, 1993; Sommer *et al.*, 1991; Koschinsky *et al.*, 1993) to form lipoprotein(a). Lipoprotein(a) particles resemble LDL particles in lipid composition. Lipoprotein(a) has recently been reviewed (Berg, 1991).

1.6.2.1.3 ApoB Function

The main function of apoB is as a structural component of plasma lipoproteins. ApoB is required for the assembly and secretion of VLDL from hepatocytes (Young, 1990). ApoB is an obligate component of VLDL, LDL and IDL, and is thought to bind lipids (see above). ApoB is also involved in the clearance of LDL, IDL and VLDL from plasma as it contains two regions of homology to the LDL-receptor binding domain of apoE (Chan, 1992). ApoB may also be involved in heparin binding, therefore directing lipoprotein particles to the site of lipases (Chan, 1992).

1.6.3 Assembly and Secretion of VLDL

The secretory pathway followed by VLDL is generally similar to other proteins (Halban and Irminger, 1994) made in hepatocytes (Dashti, 1991), except transit of particles out of the ER is rate-limiting (Borchart and Davis, 1987; Borén *et al.*, 1990). A significant proportion of apoB is bound to the ER membrane (Boström *et al.*, 1986; 1988; Wong and Pino, 1987; Davis *et al.*, 1990; Bamberger and Lane, 1988; Wilkinson *et al.*, 1992a; 1992b; Cartwright and Higgins, 1992; Du *et al.*, 1994). It is postulated that this pool of apoB is targeted to degradation (Dixon *et al.*, 1991; Furukawa *et al.*, 1992). The particles are assembled from apoB, TG, cholesterol, CE and phospholipids, which are synthesized at or on the ER (Sparks and Sparks, 1992; Borén *et al.*, 1993; Yao and McLeod, 1994). The site of assembly of full-sized VLDL particles has been localized to the luminal face of the ER (Rusiñol *et al.*, 1993a; Borén *et al.*, 1992; 1994; Dixon and Ginsberg, 1993). Pulse-chase experiments with cycloheximide show assembly of apoB100 containing VLDL is dependent upon ongoing protein biosynthesis (Borén *et al.*, 1994). A lack of TG, PC and cholesterol synthesis has been shown to block VLDL assembly and target apoB to a degradation pathway (Sakata *et al.*, 1993; Yao and Vance, 1988; Adeli, 1994; White *et al.*, 1992; Borén *et al.*, 1993; Fungwe *et al.*, 1992; Sniderman and Cianflone, 1993; Tanaka *et al.*, 1993) as has the presence of polyunsaturated (n-3) fatty acids (Wang *et al.*, 1993; 1994). Under physiological conditions, most data suggest that the rate of apoB synthesis does not regulate apoB secretion (Yao and McLeod, 1994; Pullinger *et al.*, 1989). Nascent lipoproteins can be recovered in the ER and the Golgi (Davis *et al.*, 1990; Dixon *et al.*, 1992; Rusiñol *et al.*, 1993a; Borén *et al.*, 1993; 1994) where post-translational modifications of apoB occur, after which VLDL is transported to the plasma membrane by secretory vesicles. VLDL particles are then released into the space of Disse by exocytosis (Alexander *et al.*, 1976).

1.6.3.1 Transcriptional Regulation of VLDL Assembly

Transcription of apoB is tightly regulated and is relatively constant under a wide range of metabolic conditions (Yao and McLeod, 1994). Therefore, VLDL assembly and secretion are not thought to be regulated by levels of apoB mRNA under normal conditions. However changes in apoB mRNA levels may result from changes in transcription or mRNA stability. In HepG2 cells, the half-life of apoB100 mRNA (unedited) was shown to be 16 h (Pullinger *et al.*, 1989), and thus, changes in the mRNA could not account for the rapid changes in apoB secretion observed under a variety of experimental protocols. The half-life of apoB mRNA has not been determined for normal hepatocytes or enterocytes (Sparks and Sparks, 1993). Changes in apoB mRNA levels have been observed under altered metabolic states such as hypothyroidism and starvation (reviewed by Sparks and Sparks, 1993). In transfected cells, there is a positive correlation of apoB mRNA expression with apoB and TG secretion suggesting that mRNA levels can influence VLDL secretion. However, the physiological relevance of the changes in apoB mRNA levels remains uncertain (Yao and McLeod, 1994).

However, editing of the apoB mRNA into apoB48 is a potential site of regulation, as a number of physiological factors (i.e., fasting, hormones, age) cause changes in the relative amounts of apoB48 and apoB100 produced by hepatocytes (Davidson *et al.*, 1988; Inui *et al.*, 1992; Baum *et al.*, 1990; Leighton *et al.*, 1990; Davidson *et al.*, 1990; Sjöberg *et al.*, 1992; Higuchi *et al.*, 1992).

1.6.3.2 Translational Regulation of VLDL Assembly

Translational regulation of apoB is not thought to be responsible for changes in VLDL secretion observed under most metabolic conditions. However, translational control of apoB production has been shown to occur in hypoinsulinemic diabetic rats. This is thought to involve pausing during translation (Sparks *et al.*, 1992). It has been recently observed that hepatic polysomes containing apoB mRNA have unusual physical properties (Chen *et al.*, 1993). It is possible that a critical high order structure is required for efficient translation, and that assembly of this structure is regulated by factors such as hormones (Sparks and Sparks, 1993; Yao and McLeod, 1994). The translation of apoB100 has been shown to take approximately 15 min (Boström *et al.*, 1986).

1.6.3.3 Regulation of VLDL Assembly by Translocation of ApoB

Protein translocation across the ER membrane is a process that all secretory proteins must undergo (reviewed by Lingappa, 1991; Simon and Blobel, 1991; Rapoport, 1990; Gilmore, 1993; High and Stirling, 1993). This process is directed by a signal peptide at the amino-terminus of the protein that directs the polypeptide to the translocation channel, and is cleaved upon translocation (Crowley *et al.*, 1993). Translocation of apoB across the ER membrane is a potential site for regulation of VLDL assembly and secretion.

Lingappa and coworkers have demonstrated the existence of pause transfer sequences in apoB that transiently pause the translocation of amino-terminal fragments of apoB, using *in vitro* translation/translocation assays (Chuck *et al.*, 1990; Chuck and Lingappa, 1992; Chuck and Lingappa, 1993). These pause transfer sequences are 33 amino acids in length and contain charged amino acids. They are believed to mediate the stopping and restarting of apoB translocation by interacting with a receptor associated with the aqueous translocation channel in the ER membrane (Chuck and Lingappa, 1993). The pauses in apoB translocation may be necessary for the correct folding and to allow addition of lipid to apoB (Chuck and Lingappa, 1992; Chuck and Lingappa, 1993). Further evidence for novel translocation of apoB has come from studies in which carboxyl-truncated constructs of apoB have been expressed in non-hepatic cells. When apoB53 (the amino-terminal 53% of apoB100) is expressed in hepatoma cells, apoB53 is assembled into, and secreted as a lipoprotein (Yao *et al.*, 1991). However, when apoB53 is expressed in Chinese hamster ovary cells, a non-hepatic cell line, it is incompletely translocated (Thrift *et al.*, 1992). The apoB53 expressed in Chinese hamster ovary cells is glycosylated, and therefore, at least partly translocated into the ER lumen. A lack of apoB translocation has also been shown in HeLa and COS-1 cells (Gordon *et al.*, 1994; Leiper *et al.*, 1994). The lack of translocation in non-hepatic cells may be related to lipid availability, and may reflect the co-translation addition of lipid (Borén *et al.*, 1993). Translocation of apoB constructs is observed in non-hepatic cells, if MTP is co-expressed with apoB (Gordon *et al.*, 1994; Leiper *et al.*, 1994). The action of MTP is thought to pull apoB through the membrane, by addition of lipid to the nascent apoB-containing lipoprotein.

A change in the phospholipid composition of membranes has also been shown to affect translocation of apoB constructs. When rat liver microsomal membranes are enriched in phosphatidylmonomethylethanolamine (a PC analog), apoB translocation is inhibited when measured by *in vitro* transcription translation assays (Rusiñol *et al.*, 1993b). This is supported by studies (Vance, 1991) in whole animals and cultured hepatocytes that show a decrease in VLDL secretion in the presence of monomethylethanolamine (a choline analog that is incorporated into phosphatidylmonomethylethanolamine). Studies on the translocation of apoB in choline deficiency have shown that apoB constructs are protected from exogenous protease when translocated into either CD or CS rat liver microsomes (Vermuelen, P.S., Rusiñol, A., and Vance, D.E., unpublished data).

Further evidence for translocational regulation of VLDL assembly and secretion came from computer modeling studies of apoB interaction with membranes that suggest that VLDL assembly may be translocationally controlled (Chen *et al.*, 1994).

1.6.3.4 Regulation of VLDL Assembly by Lipid Availability

Since VLDL is composed mainly of lipid, it is not unexpected that lipid availability can be limiting for the production of VLDL. Secreted apoB100 and apoB48 are 100% associated with lipids (Hussain *et al.*, 1989). ApoB100 floats in the VLDL and IDL density ranges, while apoB48 is found in all lipoprotein containing fractions ($d \leq 1.21$ g/ml). The VLDL particles produced by the liver are heterogenous in size and TG composition (Steiner and Ilse, 1981). The addition of lipids to apoB occurs cotranslationally (Boström *et al.*, 1986; 1988; Olofsson *et al.*, 1987b; Borén *et al.*, 1993). This was shown by pulse-chase analysis.

The lipids required for lipoprotein assembly and secretion are synthesized in the cytoplasmic leaflet of the ER membrane. Amphipathic molecules (phospholipids and cholesterol) can flip-flop to the luminal face of the ER membrane to be added to the growing lipoprotein particle (Olofsson *et al.*, 1987b). However TG has limited solubility in the cytoplasm and would accumulate in the hydrophobic portions of the membrane (reviewed by Lerique *et al.*, 1994). However, the amount of TG stored in this form would be insufficient to supply the needs of VLDL synthesis. Therefore, it is likely that apoB overcomes this difficulty by protruding into the lumen of the ER, creating a hydrophobic region, which could accommodate the newly synthesized neutral lipids. Chao *et al.* (1986) showed that TG was transferred from ER membranes to nascent VLDL and this regulated VLDL assembly and secretion. One possible mechanism for the loading of neutral lipid into this region is the action of MTP. In the initial stages of lipoprotein assembly, apoB cotranslationally associates with lipid (Olofsson *et al.*, 1987b; Borén *et al.*, 1992). MTP is a located in the lumen of microsomal membranes and is a heterodimer of 88 and 58 kDa subunits (Atzel and Wetterau, 1993). It catalyzes the transport of TG, CE and PC between membranes, thus suggesting that it has a role in lipoprotein assembly. Further evidence is that patients who are abetalipoproteinemic have an absence of MTP activity (Wetterau *et al.*, 1992; Shoulders *et al.*, 1993). However, the exact location and/or role of MTP in VLDL assembly is not known.

1.6.3.4.1 Triacylglycerol

Incubation of HepG2 cells with exogenous oleic acid appears to stimulate apoB containing lipoprotein secretion (Boström *et al.*, 1988; Pullinger *et al.*, 1989; Dixon *et al.*, 1991; Erickson and Fielding, 1986; White *et al.*, 1992; Borén *et al.*, 1993). This increase in apoB secretion is not observed in rat hepatocytes or liver (Petit *et al.*, 1982; Davis and Boogaerts, 1982), although TG synthesis is stimulated. The stimulation of VLDL secretion by oleate is thought to be a result of increased TG synthesis and availability (Wu *et al.*, 1994b). Extracellular fatty acids are not used directly for VLDL assembly, but rather are incorporated into a storage pool in the cytosol (Gibbons *et al.*, 1992). Three pools of TG have been observed in rat hepatocytes (Glaumann *et al.*, 1975): 1. a cytosolic pool, where TG is stored as lipid droplets, wth a relatively long half-life; 2. an ER pool with a very short half-life; and 3. an ER luminal pool, associated with lipoproteins, that has a short half-life. Oleate addition appears to stimulate turnover of the cytosolic pool of TG (Gibbons, 1994; Gibbons *et al.*, 1992; Duerden and Gibbons, 1990) partly due to stimulation of phosphatidate phosphohydrolase (Brindley, 1991). Exogenous oleate addition does not affect CE synthesis in short term experiments (Furukawa and Hirano, 1993) nor does it affect phospholipid biosynthesis (Duerden *et al.*, 1990; Borén *et al.*, 1993). Supplementation of HepG2 cells with oleate decreases the intracellular degradation of apoB (Dixon *et al.*, 1991). Studies in hamster hepatocytes (Arbeeny *et al.*, 1992) in which the synthesis of TG was blocked with an inhibitor of acetyl-CoA carboxylase, showed that apoB secretion was inhibited when TG synthesis was inhibited. These data suggest that TG availability is not normally limiting for apoB secretion in rat and hamsters, but can be so under extreme circumstances. This is in contrast to HepG2 cells where the rate of TG synthesis in the ER pool may be limiting for apoB secretion and can be stimulated by exogenous oleate addition (reviewed in Dixon and Ginsberg, 1993). Further studies on the mechanism of oleate stimulated apoB secretion in HepG2 cells suggest that the amount of lipid associated with apoB in the ER lumina determines the extent of apoB degradation (Furukawa *et al.*, 1992).

The polyunsaturated (n-3) fatty acids, eicosapentaenoate and docosahexaenoate impair VLDL secretion (Daggy *et al.*, 1987; Byrne *et al.*, 1992; Wang *et al.*, 1993; 1994; Wong *et al.*, 1989; Lang and Davis, 1990; Zhang *et al.*, 1993b), relative to oleic acid, but do not adversely affect TG biosynthesis (Lang and Davis, 1990, Wang *et al.*, 1993; 1994). In McA-RH7777 cells expressing carboxy-truncated human apoB constructs, supplementation with docosahexaenoic acid, relative to supplementation with oleic acid, reduced secretion of apoB18, apoB28 and apoB42 by 10%, 50% and 70% respectively (Wang *et al.*, 1993; 1994). They also observed that lipid-rich particles generated in the presence of exogenous n-3 fatty acids were preferentially degraded, relative to lipid-poor particles generated under the same conditions. Thus suggesting that n-3 fatty acids interfered with the correct assembly of lipid and apoB on the nascent lipoprotein particles and therefore targeted the VLDLs to a degradation pathway.

1.6.3.4.2 Cholesterol and Cholesteryl Esters

A number of studies have shown that either cholesterol or CE availability may regulate apoB secretion from hepatocytes (La Ville *et al.*, 1984; Arad *et al.*, 1990; Ginsberg *et al.*, 1987; Khan *et al.*, 1989; Khan *et al.*, 1990; Cianflone *et al.*, 1990; Fuki *et al.*, 1989; Tanaka *et al.*, 1993). CE depletion in rabbit hepatocytes decreased apoB secretion and increased intracellular apoB degradation (Tanaka *et al.*, 1993). However, other studies have shown no effect of cholesterol or CE on apoB secretion (Davis and Malone-McNeal, 1985; Wu *et al.*, 1994a; Furukawa and Hirano, 1993). CE have been shown to compete with TG for the neutral lipid core of VLDL (Davis *et al.*, 1982; Fungwe *et al.*, 1992), and this may explain some of the discrepancies observed in the importance of cholesterol and CE in VLDL secretion. A thorough review of cholesterol and CE involvement in apoB-containing lipoprotein secretion is available (Dixon and Ginsberg, 1993).

1.6.3.4.3 Phospholipids

Phospholipids are required for VLDL assembly and secretion. The major phospholipid is PC, and, as discussed above, inhibition of PC biosynthesis interferes with the secretion of VLDL. The role of the minor phospholipids (PE, lysophosphatidylcholine, phosphatidylserine, and phosphatidylinositol) in VLDL secretion has not been established. Sphingolipid biosynthesis has been shown not to be essential for VLDL secretion in rat hepatocytes (Merrill, A.H. Jr., Lingrell, S., Wang, E., Nikolova-Karakashian, M., and Vance, D.E., unpublished data). The presence of N-monomethylethanolamine, a structural analog of choline, which is incorporated into phosphatidylmonomethanolamine, in the diet of rats, or added to the medium of hepatocytes, blocks the secretion of VLDL by 50% (Vance, 1991; Rusiñol *et al.*, 1993b). Under these conditions, TG accumulates within cells. The effect of phosphatidylmonomethanolamine is due to a specific blockage of apoB translocation across the ER membrane (Rusiñol *et al.*, 1993b). The mechanism for the impaired translocation in phosphatidylmonomethanolamine enriched ER membranes may be due to changes that modify apoB interaction with the translocation apparatus. Since phospholipid biosynthesis occurs on the cytosolic face of the ER, and incorporation into VLDL occurs at the luminal face, phospholipids must traverse the bilayer (Vance and Vance, 1990a; 1990b). The translocation of phospholipids as a regulatory site in VLDL assembly has not been examined to date. Endogenous, rather than exogenous fatty acids are preferentially incorporated into phospholipids used for lipoprotein secretion (Duerden *et* *al.*, 1990). Since VLDL contains multiple components, it is conceivable that each of the components may be limiting in the production of particles.

1.6.3.5 Regulation of VLDL Assembly by Degradation of ApoB

Regardless of the amount of VLDL secretion, apoB degradation appears to be constantly occurring. Quantitative pulse-chase studies in rat hepatocytes show that a significant amount of both apoB48 and apoB100 are degraded intracellularly (Borchart and Davis, 1987). Intracellular degradation of apoB has also been observed in HepG2 cells (Adeli, 1994; Borén *et al.*, 1990; Dixon and Ginsberg, 1993; Furukawa *et al.*, 1992; Sato *et al.*, 1990). In HepG2 cells, degradation of non-secreted apoB occurs in a pre-Golgi compartment (Sato *et al.*, 1990), as it is not blocked by addition of BFA, or lysosomal protease inhibitors. Addition of oleate to HepG2 cells appeared to protect a portion of apoB from intracellular degradation by shunting apoB away from the ER membrane, i.e., into lipoprotein particles, and led to increased secretion (Dixon *et al.*, 1991; Furukawa *et al.*, 1992). Further studies in permeabilized HepG2 cells have indicated that the intracellular degradation of apoB occurs in a pre-Golgi compartment, via a calcium independent, ALLN sensitive protease that is temperature and pH sensitive (Adeli, 1994). In rat liver, proteolytic fragments of apoB have been observed by monoclonal antibody reactivity in ER, but not Golgi, suggesting that degradation of apoB occurs in the ER (Davis *et al.*, 1989). Further studies by the same group have shown that about 50% of apoB in ER is membrane bound, and susceptible to exogenous proteases (Davis *et al.*, 1990). Studies in non-hepatic cell lines have shown that apoB53 translocation into the ER lumen is impaired, resulting in stable integration of apoB53 into the membrane (Du *et al.*, 1994), the carboxy-terminal portion of this protein is degraded on the ER membrane (Thrift *et al.*, 1992), and can be blocked with ALLN. The resulting

85 kDa peptide is either released into the secretory pathway, or degraded by an ALLN sensitive proteolytic pathway (Du *et al.*, 1994). The degradation of apoB in the ER membrane is similar to the degradation of HMG-CoA reductase (Chun *et al.*, 1990; Inoue *et al.*, 1991). Degradation of both HMG-CoA reductase (Inoue *et al.*, 1991) and apoB (Thrift *et al.*, 1992; Adeli, 1994; Du *et al.*, 1994) occur on the ER, and can be blocked with ALLN. Degradation of apoB may also be compared to the α subunit of the T-cell receptor (Bonifacino *et al.*, 1990; 1991; Wileman *et al.*, 1993). The α subunit of the T-cell receptor undergoes intracellular degradation if not correctly assembled with other subunits (Wileman *et al.*, 1993). The degradation of the α subunit of the T-cell receptor is enhanced when cellular calcium is depleted (Wileman *et al.*, 1991). However, degradation of theα subunit of the T-cell receptor is not blocked by ALLN (Inoue and Simoni, 1992).

There is also thought to be a second degradation pathway that may degrade luminal apoB that has formed an HDL-like particle in the ER lumen (Borén *et al.*, 1992), although this has not been defined. Proteolysis and lipid facilitated translocation appear to be competing processes for apoB (Sakata *et al.*, 1993).

Abnormal assembly of lipid with apoB may also be responsible for targeting apoB to a degradative pathway. Fisher and co-workers have shown that in the presence of exogenous polyunsaturated (n-3) fatty acids, apoB secretion is inhibited (Wang *et al.*, 1993; 1994). Under these conditions apoB from lipid-rich particles is preferentially degraded relative to apoB on lipid-poor particles (Wang *et al.*, 1994). This degradation is thought to occur in a post-ER compartment.

ApoB48 and apoB100 do not appear to be degraded at the same rate in rat hepatocytes, as apoB48 has a half-life approximately double that of apoB100 (Sparks and Sparks, 1990b). Yao and co-workers, have examined the relationship between apoB length and stability using carboxy-terminal truncated apoBs expressed in McA-RH7777 cells (Yao *et al.*, 1991; McLeod *et al.*, 1994). There appears to be three classes of degradation; species of apoB shorter than B37 are secreted with minimal degradation, species of apoB between 42 and 60% of apoB100 have degradation patterns resembling those of apoB48, and apoB constructs longer than apoB72 have degradation patterns resembling apoB100. The authors have proposed the existence of sequences between the carboxy terminals of apoB37 and apoB42, and between apoB60 and apoB72, that are involved in mediating intracellular degradation of apoB (Yao and McLeod, 1994).

1.6.3.6 Other Factors Affecting VLDL Secretion

The rate of apoB transport from the ER of rat hepatocytes (Borchart and Davis, 1987), and HepG2 cells (Borén *et al.*, 1990) determines the rate of VLDL secretion from cells. Hence, assembly of nascent particles may limit the rate of secretion from the ER. However, in chicken hepatocytes, transit through the Golgi may be rate-limiting in the secretion of VLDL (Bamberger and Lane, 1990). The minimal transit time of most secretory proteins through the secretory pathway is 15 min (Boström *et al.*, 1986), but most apoB is retained intracellularly for longer periods of time (Borén *et al.*, 1991). The half lives of apoB100, apoB48 and albumin in rat hepatocytes are 45 min, 56 min and 28 min respectively (Borchart and Davis, 1987). In HepG2 cells the rate of secretion is as follows; apoB48 < apoB100 < albumin (Borén *et al.*, 1990).

Orotic acid supplementation causes severe fatty liver and impaired lipoprotein secretion (Windmueller *et al.*, 1964) with accumulation of lipid droplets in the cytosol (Hay *et al.*, 1988). The impaired VLDL secretion induced by orotic acid supplementation is rapidly reversed by the addition of adenine (Windmueller *et al.*, 1964). In orotic acid-treated rat hepatocytes, membrane bound apoB degradation is prevented and this pool of apoB accumulates in the *trans*-Golgi membranes (Cartwright *et al.*, 1993). However, as nascent particles can be observed in the ER lumen by electron microscopy, the defect in VLDL secretion caused by orotic acid feeding appears to be transport of nascent VLDL from the ER to the Golgi (Hamilton *et al.*, 1986). Orotic acid supplementation causes impaired fatty acid biosynthesis and increased cholesterol synthesis in rats (Tokmakjain and Haines, 1985).

Dexamethasone, a synthetic glucocorticoid stimulates VLDL secretion (Martin-Sanz *et al.*, 1990). Insulin has been shown to have varying effects on lipoprotein assembly and secretion. The role of insulin in lipoprotein assembly and secretion has been thoroughly reviewed by Sparks and Sparks (1993). Prostaglandins E_2 and D_2 inhibit secretion of VLDL, TG and cholesterol (Björnsson *et al.*, 1992b). These cells accumulate TG (without a change in synthesis rate), but not apoB. The prostaglandins are thought to affect apoB secretion via their effect on calcium levels. Studies also show that calcium (Nossen *et al.*, 1987; Björnsson *et al.*, 1992b) and copper (Nassir *et al.*, 1993) levels may modulate VLDL assembly and secretion. The effect of hormones on VLDL assembly and secretion has been recently reviewed (Björnsson *et al.*, 1992a; Sparks and Sparks, 1993).

The metabolic state of animals or isolated cells may affect the assembly and secretion of apoB containing lipoproteins. As discussed above, the availability of lipid may affect the assembly and secretion of VLDL, and thereby affect the lipid composition of secreted particles (reviewed by Sparks and Sparks, 1993; Sniderman and Cianflone, 1993). Hepatocytes from fasted rats showed decreased TG synthesis and secretion resulting in smaller VLDL particles (Davis *et al.*, 1985), and hepatocytes from sucrose fed rats showed increased VLDL synthesis and secretion (Boogaerts *et al.*, 1984). Studies in humans have shown that TG availability influences the size of VLDL secreted by the liver (Ginsberg *et al.*, 1985). Under the same conditions, apoB48 synthesis was decreased, but apoB100 synthesis was unchanged. This suggests that apoB100 and apoB48 do not always respond in the same manner to a stimulus. High levels of glucose or free fatty acid also appeared to stimulate VLDL secretion (Arrol *et al.*, 1991). However, high levels of amino acids have a negative regulatory effect on apoB synthesis and secretion (Zhang *et al.*, 1993a), and regulation of secretion in HepG2 cells was shown to be inversely correlated with medium albumin concentration (Pullinger *et al.*, 1989; Cianflone *et al.*, 1994).

Proper folding and the action of chaperones (Hartl *et al.*, 1992; 1994; Ellis and Vandervies, 1991; Craig, 1993) such as protein disulfide isomerase (Noiva and Lennarz, 1992) may be important in the assembly of apoB into a VLDL particle. Protein disulfide isomerase is a subunit of MTP (Wetterau *et al.*, 1990; 1991a; 1991b). MTP is thought to be involved in the assembly of lipid with nascent particles, and the protein disulfide isomerase component of this complex may be involved in holding apoB in the correct conformation for the addition of lipid. In patients with abetalipoproteinemia there is a lack of MTP activity (Wetterau *et al.*, 1992; Shoulders *et al.*, 1993; Leiper *et al.*, 1994). Another condition in which VLDL secretion is impaired is familial hypobetalipoproteinemia. This is an autosomal co-dominant disorder characterized by low or absent levels of apoB containing lipoproteins in plasma. This disease is caused mainly by mutations in the gene for apoB, often causing truncated apoB species to be produced. The symptoms and causes of familial hypobetalipoproteinemia have recently been reviewed (Linton *et al.*, 1993).

1.6.4 Models of VLDL Assembly

ApoB is synthesized in excess and is targeted to the ER membrane by its signal sequence where it translocates through the membrane (Dixon and Ginsberg, 1993). The apoB is then associated with lipids by one of the three possibilities listed below. If the addition of lipid to the particle is not achieved due to lack of substrate, incorrect positioning of the apoB, or some other factor, the apoB integrates into the ER membrane and is targeted to a degradation pathway that can be blocked with ALLN (Thrift *et al.*, 1992; Du *et al.*, 1994; Adeli, 1994). It is also possible for the particle to be only partially assembled with lipid (Boström *et al.*, 1988; Borén *et al.*, 1993) and form an apoB containing particle of HDL density. This HDL-like particle may be targeted to an alternate degradation that has not been defined, or it may undergo a subsequent addition of lipid and be secreted (Borén *et al.*, 1994).

If the required components of VLDL are not limiting, there are three models for apoB association with lipids in hepatocytes. The three different models have arisen due to different model systems used to study the process of VLDL assembly. The starting material for these studies includes rat and chicken hepatocytes, rat liver, and human hepatoma cells. Many of the studies have used subcellular fractionation techniques to isolate nascent VLDL, often after pulse-chase protocols. Variation in the recovery, purity and characterization of the subcellular fractions from the various cell types complicates the interpretation and has led to the three models of VLDL assembly presented here.

1.6.4.1 Endoplasmic Reticulum Assembly Model

Nascent VLDL particles are formed completely in the ER, and are only modified in the Golgi, but further addition of lipid does not occur. In this model, apoB is synthesized on ribosomes attached to the rough ER. This must all take place at or near the site of synthesis of phospholipid, TG, cholesterol and CE so that co-translational addition of lipid to apoB can occur (Boström *et al.*, 1986; 1988; Olofsson *et al.*, 1987b; Borén *et al.*, 1990). The ER is the main site of synthesis of these compounds. The lipid loading of the particle may be aided by MTP or other proteins not yet identified. Once the particle has reached the correct size that allows the apoB to fold correctly, it buds from the membrane and rapidly enters the Golgi. The apoB-containing VLDL are then secreted by the normal secretory route. This model is supported by studies that have observed apoB containing nascent VLDL particles in the lumen of the ER by immunoelectron microscopy (Alexander *et al.*, 1976), pulse-chase studies in rat hepatocytes (Borchart and Davis, 1987; Cartwright *et al.*, 1993) and cultured human hepatoma cells (Borén *et al.*, 1992), and by lipid analysis of apoB containing particles from the ER lumen isolated by immunoaffinity chromatography (Rusiñol *et al.*, 1993a). The size and composition of VLDL particles isolated from the ER lumen was very similar to VLDL isolated from plasma or culture medium from rat hepatocytes (Rusiñol *et al.*, 1993a). This is the most likely model of VLDL assembly.

1.6.4.2 Golgi Assembly Model

In this model, apoB is synthesized on the rough ER membrane and remains membrane associated until apoB reaches the Golgi. Then the apoB combines with neutral and phospholipids and is released into the Golgi lumen as VLDL and then secreted. This model is supported by pulse-chase experiments in estrogen-treated chicken hepatocytes (Bamberger and Lane, 1990) and rat liver (Higgins, 1988; Cartwright and Higgins, 1992), and by immunoblot studies of rabbit liver subcellular fractions (Wilkinson *et al.*, 1990; 1992a). Assembly of VLDL via this model is unlikely as Rusiñol *et al.* (1993) have isolated nascent VLDL from the ER lumina of rat liver with the same size and lipid composition as plasma VLDL. The reason for the discrepancy may be related to the model system used, as discussed above.

1.6.4.3 Two Step Model

In this model, VLDL is formed in two or more discrete steps. ApoB is translated on ribosomes attached to the ER membrane as in the other two models and combines with some lipid in the ER to form an apoB containing particle of HDL density. As this particle moves through the ER and Golgi, lipid is added sequentially. This work is supported by pulse-chase studies in chicken (Janero and Lane, 1983) and rat (Boström *et al.*, 1986; 1988; Elovson *et al.*, 1992; Borén *et al.*, 1990; 1994) hepatocytes. This model is also plausible since Golgi membranes do have the capacity to synthesize phospholipids (Vance and Vance, 1988). However, this model is not the most likely for the assembly of VLDL as immunoelectron microscopy does not provide evidence for increasing particle size as the nascent particle moves through the ER to the Golgi (Alexander *et al.*, 1976; Rusiñol *et al.*, 1993a). However, in rat hepatocytes where apoB48 is used for VLDL assembly, and in chylomicron assembly in the intestine, such a model may be required to explain the large amount of lipid per apoB molecule. Borén *et al.* (1994) have shown using pulse-chase experiments that apoB48 in the lumen of microsomes resides on HDL-like particles. Over time, a portion of this pool is converted to an apoB48 containing VLDL particle in McA-RH7777 cells, and is secreted.

Regardless of the models presented here, VLDL assembly is likely a complex multi-step process as many components are required for the proper synthesis and secretion of VLDL. Also, none of the models exclude an exchange of phospholipid (Janero and Lane, 1983) or neutral lipid (Howell and Palade, 1982) between the nascent VLDL and the subcellular organelle membranes.

1.6.5 Catabolism of Plasma VLDL

The catabolism of VLDL begins almost immediately after entry of the particles into the plasma and is very rapid. Lipoprotein lipase, which is attached to the surface of endothelial cells, associates with the coat of the particles. The hydrolysis of the TG core by lipoprotein lipase is activated by apolipoprotein C-II (reviewed by Braun and Severson, 1992; Wang *et al.*, 1992; Enerbäck and Gimble, 1993). As the TG is hydrolyzed, the particle becomes smaller (IDL or LDL sized), and there is an exchange of apolipoproteins. In the rat, apoB100-containing VLDL have a longer half life in plasma than do apoB48-containing VLDL (Elovson *et al.*, 1981). This may be due to increased amounts of apoE on apoB48 containing particles, so that they are more efficiently cleared by the LDL-receptor (Davis, 1991). Catabolism of VLDL and LDL has recently been reviewed (Griffin and Packard, 1994).

1.7 Summary of Approach and Results

In this thesis, I have extended the earlier studies of choline deficiency (Yao and Vance, 1988; 1989; 1990) to define the site of the defect in VLDL secretion. This was accomplished by subcellular fractionation of rat liver and subsequent detection of apoB. I demonstrated that the number of apoB containing particles in the ER is equivalent between CD and CS samples, but in the Golgi from CD rat livers the levels of apoB are reduced to approximately 50% of the CS values. I also examined the secreted particles in both CD and CS states and showed that the CD particles are larger and contain less PC and relatively more PE. ApoB on CD and CS plasma VLDL have different conformations as shown by protease digestion. I have also been able to characterize the lipid composition of the nascent particles in the lumen of the ER and Golgi, as well as the membranes of these organelles. Nascent VLDL particles isolated from the lumen of the CD secretory pathway are deficient in PC and have a relative enrichment in PE. The phospholipid composition of the nascent particles resembles the membrane phospholipid composition suggesting that phospholipids may exchange between nascent lipoproteins and the membranes of the secretory organelles. I have also characterized the rate of secretion of bulk proteins and shown that the rate of protein secretion is decreased in CD compared to CS hepatocytes although, not to the same extent as VLDL secretion. Moreover, I have shown that VLDL particles are not degraded when secretion was blocked at the level of the ER. The model proposed by this work is that when PC biosynthesis is decreased, nascent VLDL is assembled incorrectly (i.e., has a larger particle size and abnormal lipid composition) and therefore is recognized and degraded by a putative quality control protease in a post-ER compartment.

2. Materials and Methods

2.1 Chemicals

[^{3}H]-Leucine (73 Ci/mmol), Pro-Mix™ [^{35}S] cell labeling mix (> 1000 Ci/mmol), [^{125}I]-sodium iodide (100 Ci/ml, UDP [6-^{3}H]-galactose (14.5 Ci/mmol) and enhanced chemiluminescence detection kit were purchased from Amersham Corp. Goat anti-rabbit horseradish peroxidase conjugate, Iodogen™ and BCA protein assay reagent were from Pierce Laboratories. The reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories. PVDF membranes (Immobilon P) were from Millipore. DMEM was from Gibco. The Triglyceride-G kit for quantitation of TG was obtained from Wako Pure Chemical Industries Ltd., Japan. Thin-layer chromatography plates (Silica Gel G, 0.25 mm thickness) were purchased from BDH Chemicals, and standard lipids were from either Avanti Polar Lipids or from Sigma. All other chemicals were from Sigma or Fisher.

2.2 Antibodies

Antibodies against VLDL or albumin were raised in rabbit by conventional techniques. The antigen (100 µg) was injected subcutaneously at 5 or 6 sites on the back of the neck of a New Zealand white rabbit, initally with complete Freund's adjuvant followed 5 to 6 weeks later by antigen in incomplete Freund's adjuvant (Hurn and Chantler, 1980). Two weeks after the boost, blood was drawn from the ear vein, incubated at 37 °C for 2 h and the serum isolated by centrifugation (20 min at 50 x *g*). Antisera were stored at -70 °C in 0.5 ml aliquots containing 0.1% (w/v) NaN_3. Specificities of the antibodies were confirmed by SDS-PAGE and subsequent immunoblotting of rat plasma VLDL or albumin respectively. The antibodies were also tested for quantitative immunoprecipitation of apoB or albumin by sequential immunoprecipitations of ^{125}I-VLDL or labeled cells and medium.

2.3 Animals and Diet

Choline-deficient diet (ICN Biochemicals, Canada) consisted of 10% vitamin-free casein, 10% alpha-protein, 20% lard, 56% sucrose, 4% salt mixture (Wesson), and ICN vitamin fortification mixture that lacked choline chloride. Male Sprague-Dawley rats, initially weighing 35-45 g, were fed either this diet (CD rats) or this diet supplemented with 0.4 g choline chloride /100 g diet (CS rats) for three days. The liver and body weights were identical in CD and CS rats.

2.4 Enzyme Assays

2.4.1 NADPH:Cytochrome *c* Reductase

NADPH:cytochrome *c* reductase activity was determined according to Ernster *et al.* (1962) using 25 - 50 µg of protein from ER-I, ER-II, or Golgi subcellular fractions resuspended in buffer (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 0.1 mM PMSF). The reaction was 3 min at room temperature and followed kinetically in a Shimazdu spectrophotometer at 550 nm.

2.4.2 UDP-Galactose: N-Acetyl Glucosamine Galactosyltransferase

UDP-galactose: N-acetyl glucosamine galactosyltransferase activty was determined according to Bergeron *et al.* (1973) using ER-I (100 µg), ER-II (100 µg), or Golgi (30 µg) resuspended in buffer (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 0.1 mM PMSF). N-acetylglucosamine was used as an acceptor for galactose from UDP [6-^{3}H] galactose.

2.4.3 Lactate Dehydrogenase Assay

Lactate dehydrogenase activity was determined according to Amador *et al.* (1963). Either 10 µl of one dish of hepatocytes (resuspended in 2ml of water, sonicated for 10 s in a heat systems ultrasonic processor, setting 1), or 20 µl of medium (after removing any cells by centrifugation, 10 min at 1000 x g) were used for the assay. The reaction was performed in microtiter plates and NAD^+ formation monitored at 340 nm in an EAR 340AT microplate reader (SLT Lab Instruments).

2.5 Hepatocyte Isolation

Hepatocytes were isolated from the livers of CD rats as previously described (Yao and Vance, 1988) and plated in 60 mm dishes at a density of 3 X 10^6 cells/dish. The cells were plated in choline and methionine deficient DMEM containing 20% delipidated fetal bovine serum. After 4 h, the media were changed and all cells were incubated in the absence of serum. At this time, some dishes were supplemented with 100 µM choline (CS hepatocytes), while others remained in CD medium (Yao and Vance, 1988). After an overnight incubation (18 h) the cells were treated as described in the Figure legends.

2.5.1 Delipidation of Fetal Bovine Serum

Fetal bovine serum was delipidated with butanol/di-iso-propyl ether (40:60 v/v) by the method of Cham and Knowles (1976), and dialysed against several changes of 0.9% NaCl (w/v) to remove residual organic solvents and small molecules such as choline. The delipidated serum was sterilized by passing through a 0.2 µm filter and then stored at -20 °C in aliquots.

2.6 Subcellular Fractionation

The CD and CS rats were anaesthetized with diethyl ether and then by intraperitoneal injection of pentobarbitone (5 mg/100 g body weight). Rats were then killed by exsanguination and the livers excised. Typical liver weights of rats from either diet group was 2.0 to 2.5 g, and up to six livers were combined for subcellular fractionation. The method of Croze and Morré (1984) was used for subcellular fractionation, with the modifications as described by Vance (1990b) and by Hamilton *et al.* (1991) in order to diminish endosomal contamination of the Golgi fraction. These procedures resulted in isolation of a heavy ER fraction (ER-I), a light ER fraction (ER-II), and Golgi from CD and CS livers. In some cases the material from ER-I and ER-II was combined to give a total ER fraction. Luminal contents of the subcellular fractions were prepared by treating either ER or Golgi vesicles with 100 mM Na_2CO_3 (pH 11.5) for 30 min on ice at a protein concentration below 2 mg/ml (Fujiki *et al.*, 1982). The contents and membranes were separated by centrifugation for 50 min at 150,000 x *g*. The luminal contents were neutralized with dilute acetic acid and used for lipoprotein isolation or immunoblotting. The membrane fraction was resuspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, to a protein concentration of approximately 1 mg/ml.

Microsomes were prepared from hepatocytes (Davis *et al.*, 1990), by scraping the cells into 2 ml of phosphate buffered saline that contained 1 µM PMSF, 50 µg/ml leupeptin, and 1 µg/ml aprotinin. The cells were pelleted by centrifugation (10 min at 1000 x *g*) and resuspended in 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose. Cells were broken by 10 up and down strokes of a Potter-Elvejhem homogenizer. A total microsomal fraction was then prepared by centrifuging for 10 min (10,000 x g), the supernatant from this spin was then layered onto a 2 M sucrose cushion (2 M sucrose, 10 mM Tris-HCl, pH 7.4) and centrifuged at 105,000 x *g* for 90 min. The microsomal fraction at the 0.25 M/2 M interface was collected.

2.7 Lipoprotein Isolation

2.7.1 Isolation of Lipoproteins from Plasma

Rats (300-350 g) were fasted overnight and lightly anesthetized with diethyl ether. Blood was drawn by cardiac puncture using a 30 ml syringe with an 18 gauge needle. The samples were adjusted to 1 mg/ml EDTA, and plasma isolated by centrifuging at 500 x *g* for 20 min. The plasma was subjected to sequential floatation (Schumaker and Puppione, 1986) to obtain VLDL after a brief centrifugation (20 min at 40,000 x *g*) to remove chylomicroms. The VLDL were re-centrifuged in NaBr solutions of d = 1.006, and the purified VLDL were used for raising antibodies or as apolipoprotein standards on SDS-PAGE.

In experiments with small animals (CD and CS rats), where small amounts of plasma were obtained, lipoproteins were fractionated by the method of Brousseau *et al.* (1993) into; bottom fraction (d> 1.21 g/ml), HDL (d= 1.21 g/ml), and LDL + VLDL (d< 1.06 g/ml) by sequential density gradient ultracentrifugation. In some cases lipoproteins were concentrated onto 20 mg of Cab-O-Sil™ (Vance *et al.*, 1984). The lipids were extracted from the Cab-O-Sil™ with 5 ml chloroform: methanol (2:1), and the apoproteins were separated by SDS-PAGE as described below after resuspension in lipoprotein sample buffer.

2.7.2 Isolation of Lipoproteins from Subcellular Fractions

The luminal contents of ER or Golgi were adjusted to contain 0.02% w/v EDTA, 0.02% w/v NaN_3, and 0.1 mM PMSF and then loaded on top of 0.4 g sucrose in a polyallomer tube, overlaid with 1.3 ml NaBr (d= 1.02 g/ml). The tube was filled to the top with 0.4 M NaCl. The samples were centrifuged for 42 h at 15 °C at 150,000 x *g* in a Ti70 rotor (Rusiñol *et al.*, 1993a). Six 6.5 ml fractions were collected from the bottom of the tube. The density of each fraction was determined by refractometry or weighing. The lipoproteins were concentrated by addition of 20 mg Cab-O-Sil™ to each fraction (Vance *et al.*, 1984). The lipids were extracted from the Cab-O-Sil™ with 5 ml chloroform: methanol (2:1), and the apoproteins were resuspended in lipoprotein sample buffer and separated by SDS-PAGE as described below.

2.7.3 Iodination of VLDL

Plasma VLDL from control rats was iodinated using the reagent Iodogen™. One mg of Iodogen™ was coated onto a glass vial, then plasma VLDL added, then 1 mCi of [^{125}I] NaI was added and allowed to incubate for 20 min with gentle shaking. The reaction was stopped by separating the unreacted iodine from the VLDL by chromatography on a Bio-Gel P10 column. The first peak of radioactivity was pooled and dialysed against multiple changes of 0.9 M NaCl. The labeled apolipoproteins were then separated by SDS-PAGE, and used for quantitation of immunoprecipitation or transfer to PVDF membranes.

2.8 Intracellular Transport Inhibitor Studies

2.8.1 Brefeldin A

After preparation of hepatocytes as described above, CD and CS hepatocytes were pulse-labeled for 30 min with [^{35}S] cell labeling mix (100 µCi/dish in 1 ml DMEM). The cells were washed twice with phosphate buffered saline and radioactivity was chased for a further 90 min in the presence or absence of 5 µg/ml BFA. The media and cells were harvested, and VLDL and albumin were immunoprecipitated as described below. Proteins were separated by SDS-PAGE as described below. The bands corresponding to apoB100, apoB48 and albumin were excised from the gel and radioactivity was determined.

2.8.2 Monensin

After preparation of hepatocytes as described above, CD and CS hepatocytes were pulse-labeled for 30 min with [^{35}S] cell labeling mix (100 µCi/dish in 1 ml). The cells were washed twice with phosphate buffered saline and radioactivity was chased for a further 90 min in the presence or absence of 10 µg/ml monensin. The cells and media were harvested and VLDL and albumin were immunoprecipitated as described below. Proteins were separated by SDS-PAGE as described below. The bands corresponding to apoB100, apoB48 and albumin were excised from the gel and radioactivity was determined.

2.9 Protease Inhibitor Studies

After preparation of hepatocytes as described above, CD and CS hepatocytes were labeled for up to 16 h with [^{35}S] cell labeling mix (15 µCi/dish in 2 ml DMEM) plus the presence or absence (control) of protease inhibitors as described in the figure legends. Protease inhibitors (1000x final concentration) were dissolved in water, except for ALLN which was dissolved in 50% (v/v) ethanol. The media and cells were harvested, and VLDL were immunoprecipitated as described below. Proteins were separated by SDS-PAGE as described below and radioactivity detected by fluorography.

2.10 SDS-PAGE

2.10.1 Sample Buffers

Proteins were denatured by boiling for 5 min in buffer containing either 8 M urea, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 10 mM Tris, pH 6.8 (lipoprotein sample buffer), or 6.25 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.02% Bromophenol Blue (SDS-PAGE sample buffer).

2.10.2 Electrophoresis

Electrophoresis was on 3-15% polyacrylamide gels that contained 0.1% SDS. The proteins were first concentrated through a 3% stacking gel. Electrophoresis was for 16 h at 15 milliamperes per gel.

2.10.3 Detection of Proteins after SDS-PAGE

After electrophoreis, proteins were stained with either Coomassie Blue R-250, silver stained according to Hochstrasser *et al.* (1988) or transferred to PVDF as described below. In some cases, after staining gels were incubated for 15-20 min in Amplify™ (Amersham) and then dried overnight. Gels were exposed to film at -70°C for 4 to 96 h. For immunoprecipitation studies the apoB48 and apoB100 were identified by comparison with standard rat serum VLDL, prepared as previously described (Schumaker and Puppione, 1986), and albumin was identified by comparison with rat albumin (Sigma). In some cases, the bands containing apoB or albumin were excised from the gel, digested by heating in 0.6 ml of a mixture of 30% hydrogen peroxide and 60% perchloric acid (2:1) for 16 h at 60 °C, and radioactivity measured.

2.11 Detection of Protein by Immunoblotting

In some experiments, after electrophoresis proteins were transferred to PVDF membranes (18 h, 70 V) in 62.5 mM boric acid buffer (pH 8.0) at 4°C and subsequently reacted with rabbit polyclonal anti-rat VLDL antiserum and peroxidase-linked anti-rabbit IgG. Visualization of immunoreactive species was via enhanced chemiluminescence detection reagents.

Densitometric analysis of immunoblots was done with a CAMAG TLC Scanner II at 460nm.

Transfer of apoB100 and apoB48 from the gel to the PVDF membrane was greater than 90% as determined by transfer of ^{125}I labeled VLDL after SDS-PAGE.

2.12 Immunoprecipitations

Albumin and apoB were specifically and quantitatively immunoprecipitated as described by Borchart and Davis (1987) using either 35 µl of rabbit anti-rat VLDL or 35 µl of rabbit anti-rat albumin for one ml of medium or 1/5 of a dish of cells. Immune complexes were precipitated upon the addition of 100 µl of Immuno-Precipitin (Gibco BRL). The proteins were solubilized from the immunoprecipitate by boiling for 5 min in a lipoprotein sample buffer and separated by SDS-PAGE as described above.

2.12.1 Quantitation of Immunoprecipitations

Protein detection was by fluorography of dried gels as described above. Bands corresponding to samples of interest were excised from the gel and dissolved by incubation in 20% perchloric acid (final concentration), 20% hydrogen peroxide (final concentration) at 65°C for 12 h. Ten ml of aqueous counting scintillant (Amersham) was added, samples allowed to chemiluminesce and counted in a Beckman liquid scintillation counter.

2.13 Protein Analysis

Protein was determined with the BCA protein detection reagent (Pierce) in the presence of 0.04% (w/w) deoxycholate. Bovine serum albumin was used as a standard.

2.14 Lipid Analysis

Lipids were extracted from membranes or cells by the method of Bligh and Dyer (1959). When lipoproteins were concentrated onto Cab-O-Sil™, lipids were extracted with chloroform:methanol (2:1). Phospholipid and neutral lipid species were separated by thin-layer chromatography using a two solvent system as previously described (Yao and Vance, 1988). Lipids were detected by staining with iodine, and bands corresponding to appropriate standards were scraped from the plate. Phosphorous was measured by the method of Rouser *et al.*, (1966) after digestion of with perchloric acid (70%) at 180°C for 1 h. TG was measured by the Wako Triglyceride G kit. Cholesterol and CE were measured with the Cholesterol 20 kit (Sigma).

2.15 ELISA

2.15.1 Albumin

The amount of albumin was determined by ELISA using rat albumin as standard and a rabbit anti-rat albumin primary antibody on Immulon™ microtitre plates using between 50 and 400 µg total protein per well. The second antibody was sheep anti-rabbit peroxidase conjugate. Detection was by the peroxidase substrate ABTS (2,2′-azino-di-(3-ethylbenzthiazolinesulfonate) with detection at 405 nm.

2.15.2 ApoB

Enzyme linked immunosorbent assays were performed as described by Rusiñol *et al.* (1993a). Luminal contents were coated on Immulon microtiter plates. Polyclonal rabbit anti-rat apoB and peroxidase-linked anti-rabbit IgG were used as primary and secondary antibodies, respectively. Detection was by the peroxidase substrate ABTS (2,2′-azino-di-(3-ethylbenzthiazolinesulfonate) with detection at 405 nm.

2.16 TCA precipitations

CD or CS hepatocytes, which had been incubated overnight in leucine-free medium, were labeled for 30 min with [^{3}H]-leucine (25 µCi/dish in 1 ml). Following the pulse, the cells were washed and the radioactive medium replaced by medium containing 8.0 mM leucine for indicated chase times. Duplicate dishes of cells were harvested, lysed by sonication (Heat Systems

Ultrasonic processor, setting 1), and used for TCA precipitation. Media collected at each time point were centrifuged (10,000 x *g*) for 2 min to remove any cell debris. Proteins were precipitated from aliquots of cells and media by addition of ice cold 10% TCA (final concentration) in the presence of 1 mg carrier protein (bovine serum albumin). TCA pellets were washed three times with ice cold 5% TCA, then solubilized in 1 ml of 1.0 M NaOH. Radioactivity was measured.

2.17 Electron Microscopy of VLDL

VLDL was isolated from plasma and Golgi lumina of both CD and CS rats as described above. Immediately after preparation, the VLDL was allowed to adhere to hydrophillic carbon films and then washed with 2% sodium phosphotungstate as a negative stain. Electron micrographs were obtained with a Phillips EM420 electron microscope operated at 100 kilovolts.

2.18 Data analysis

Values are given as means ± S.E.M, or as means ± S.D. Statistical significance was determined by the student's t-test.

3 Results

3.1 Recovery of Marker Enzymes from Subcellular Fractions of Rat Liver

We used isolated subcellular fractions from rat liver (Croze and Morré, 1984). This protocol leads to two ER fractions; ER-I and ER-II, and a Golgi fraction. ER-I is enriched in rough ER, and ER-II is enriched in smooth ER (Croze and Morré, 1984). Typically, the amount of protein recovered in these fractions was 2.0-2.5 (ER-I), 1.5-2.0 (ER-II) and 0.4-0.6 (Golgi) mg per g liver, from either CD or CS livers.

3.1.1 Marker Enzymes

We examined the levels of marker enzymes for smooth ER (NADPH-cytochrome *c* reductase) and *trans*-Golgi (UDP-galactose: N-acetyl-glucosamine galactosyltransferase) in the three fractions isolated from CD and CS rat liver. The distribution pattern of these enzymes was similar in CD and CS ER and Golgi fractions as shown in Figures 4 and 5. The specific activity of NADPH-cytochrome *c* reductase was enriched in the ER-II fraction relative to ER-I or Golgi (Figure 4). UDP-galactose: N-acetylglucosamine galactosyltransferase specific activity was high in Golgi fractions and low in both ER-I and ER-II fractions (Figure 5). According to specific activity comparisons of these marker enzymes, contamination of Golgi with NADPH-cytochrome *c* reductase was 14.0 ± 1.4% (CD) and 18.3 ± 2.4% (CS). Contamination of ER-I fractions with the Golgi marker enzyme was 4.7 ± 0.6% (CD) and 4.1 ± 0.5% (CS). Similarly low contamination of ER-II fractions by UDP-galactose: N-acetylglucosamine galactosyltransferase was observed.

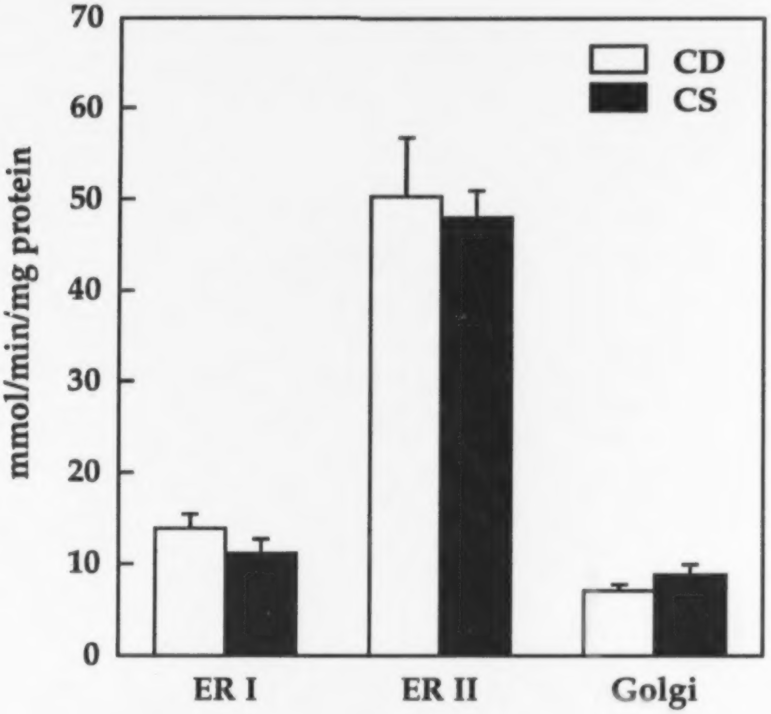


Figure 4. Specific Activity of NADPH:Cytochrome c Reductase in Subcellular Fractions from CD and CS Rat Livers

Subcellular fractions were isolated from rat liver and assayed for NADPH:cytochrome c reductase. Values represent means ± S.E.M. from four to six fractionations.

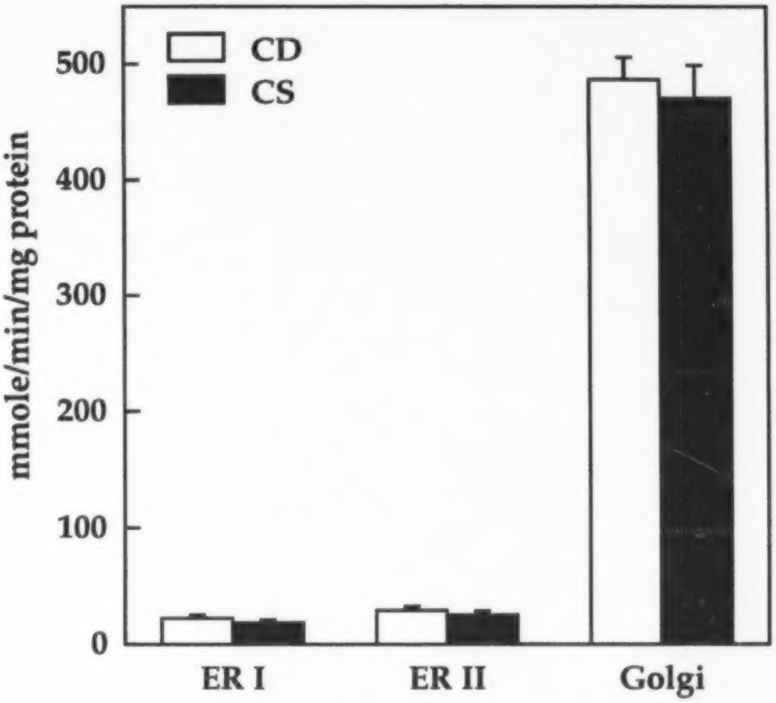


Figure 5. Specific Activity of UDP-Galactose:N-Acetylglucosamine Galactosyltransferase in Subcellular Fractions from CD and CS Rat Livers

Subcellular fractions were isolated from rat liver and assayed for UDP-galactose:N-Acetylglucosamine galactosyltransferase. Values represent means ± S.E.M. from four to six fractionations.

3.1.2 Endosomal Contamination

Contamination of Golgi by endosomes is a common problem in subcellular fractionation, and this was avoided by washing the Golgi in an excess of buffer (Hamilton *et al.*, 1991). The degree of contamination of Golgi from CD and CS rat livers by endosomes has been examined (Verkade *et al.*, 1993). The percentage of contamination in the Golgi of endogenously synthesized apoB with endocytosed apoB was 8.4% (CD) and 2.5% (CS). The contamination of ER-I and ER-II by endosomes was also low (CD ER-I, 3.2%; CD ER-II, 2.8%; CS ER-I, 4.4%; CS ER-II, 6.3%). Thus, endosomal contamination of Golgi was not a significant factor for the studies described in this thesis.

3.2 ApoB Levels in ER and Golgi Lumina and in Plasma

Initially, it was thought that particles would not be formed in choline deficiency, therefore, we expected to observe fewer particles in lumina of both ER and Golgi fractions from CD, than from CS rat livers. However, apoB is decreased in the lumina of the Golgi, but not the ER in CD compared with CS rat livers. The levels of apoB100 and apoB48 in the lumina of ER-I, ER-II and Golgi were determined by immunoblotting (Figure 6). The levels of both apoB100 and apoB48 in the lumina of ER-I and ER-II were not significantly different between CD and CS fractions. However, in the lumina of the Golgi from CD rats the amounts of both apoB100 and apoB48 were significantly reduced compared to samples from CS rats. Immunoblots from six different preparations of Golgi and three different preparations of ER-I and ER-II from CD and CS rats were analyzed by scanning densitometry (CAMAG TLC Scanner at 460 nm). The results (Table III) show that apoB48 and apoB100 levels were not significantly different between the lumina of

Figure 6. Immunoblot Detection of ApoB in Lumina of Subcellular Fractions from CD and CS Rat Livers.

Subcellular fractions were prepared from rat liver. Luminal contents corresponding to 50 µg Golgi, ER-I or ER-II protein were electrophoresed on a 3-15% polyacrylamide gel, containing 0.1% SDS. The gel was electroblotted onto a PVDF membrane, and incubated with rabbit anti-rat apoB antibody, followed by peroxidase-conjugated anti-rabbit IgG. The proteins were detected by enhanced chemiluminescence assay. No choline in the rat diet is indicated by - and + indicates that there was choline in the diet. The experiment was repeated 3 times with similar results.

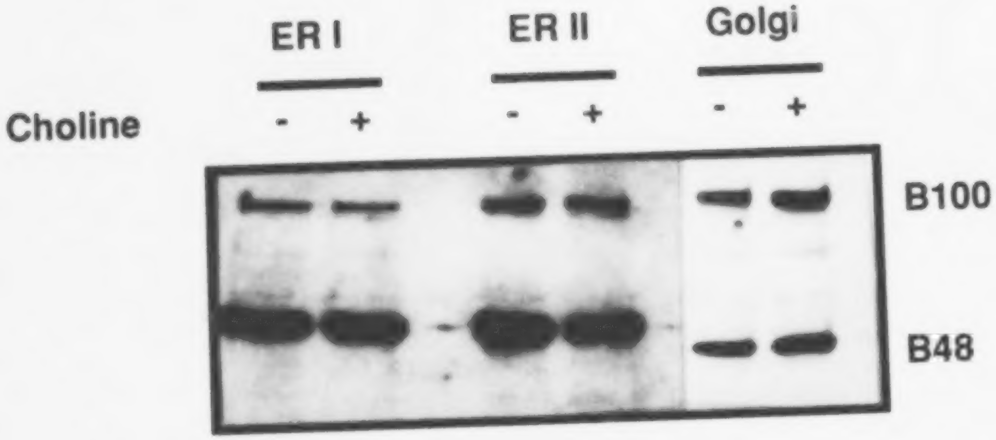

ER I
ER II
Golgi
Choline
-
+
-
+
-
+
B100
B48

Table III

Ratio of CD to CS ApoB and Albumin in Subcellular Fractions

The luminal contents of subcellular fractions were prepared from CD and CS rat liver as described in "Materials and Methods". The protein content of the luminal contents was measured, and the levels of apoB, or albumin were measured by ELISA with either rabbit anti-rat apoB, or rabbit anti-rat albumin as the primary antibody. Detection was via a peroxidase-conjugated second antibody and ABTS substrate (2,2'-axino-di-[3-ethylbenzthiazoline] sulfonate). Values for CS albumin levels per μg of lumenal organelle protein were 26.9 ng (ER-I), 11.1 ng (ER-II), and 150 ng (Golgi), and in plasma, 354 ng/μg of total plasma protein. The values for apoB ELISA were taken from Figure 7. For studies involving densitometric analysis of apoB100 and apoB48, appropriate bands were scanned with a CAMAG TLC Scanner II at 460nm. Immunoblots from 6 different preparations of Golgi, and three different preparations of ER-I and ER-II from CD and CS rats were scanned. Results are the means ± S.D. of at least 3 separate experiments.

	ELISA	Densitometry		ELISA
Fraction	Albumin	ApoB100	ApoB48	ApoB[b]
ER-I	1.28 ± 0.34	1.46 ± 0.48	1.06 ± 0.10	1.00 ± 0.22
ER-II	1.36 ± 0.25	1.13 ± 0.10	1.18 ± 0.09	1.42 ± 0.22
Golgi	1.06 ± 0.34	0.52 ± 0.29[a]	0.61 ± 0.29[a]	0.58 ± 0.09[a]
Plasma	0.99 ± 0.01	nd	nd	0.42 ± 0.05[a]

[a] $p<0.02$

[b] total apoB detected with anti apoB antibody

nd= not determined

CD and CS ER. However, levels of apoB48 and apoB100 were reduced to approximately 60% in CD relative to CS Golgi lumina. The total apoB mass in the lumina of ER and Golgi was measured by an ELISA, using a rabbit polyclonal antibody directed against rat apoB. The results (Figure 7) showed minimal differences in the luminal content of ER-I and ER-II fractions, but did show a significant ($p< 0.05$) decrease in the apoB content of the lumina of Golgi and plasma from CD relative to CS rats. These data support the hypothesis that the initial steps of VLDL assembly in the ER are not quantitatively impaired in CD livers. Since there was not an accumulation of apoB100 or apoB48 in the ER lumina of CD rats, the data also support the hypothesis that when PC biosynthesis is inhibited, there is increased degradation of apoB containing particles prior to arriving in, or within the Golgi. Alternatively, the apoB may be transferred to another subcellular fraction within the cell or lose its immunoreactivity.

3.3 Density Distribution of ApoB in ER and Golgi Lumina

We next examined the apoB distribution in nascent lipoproteins from the ER and Golgi lumina by immunoblotting. Proteins were detected by rabbit anti-rat VLDL (Figure 8). We found relatively more apoB48 in the bottom fraction ($d> 1.01$ g/ml) than in the top fraction ($d< 1.01$ g/ml) from ER for both CD and CS samples, suggesting the presence of only partially formed VLDL in the ER. Virtually all the apoB100 in the ER in both CD and CS samples was in the top or VLDL density range, suggesting that apoB100 nascent lipoproteins may be fully formed in the ER lumen. In Golgi lumina, more apoB48 was in the VLDL density range ($d< 1.01$ g/ml) for both CD and CS samples compared to ER lumina. Either more of the heavier particles are selectively degraded prior to their arrival in the Golgi, or are transformed into "VLDL" type particles by lipid addition. The rationale for the relative

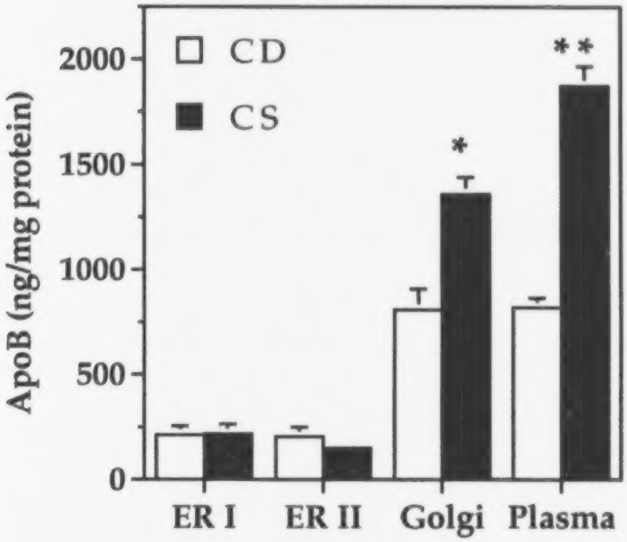
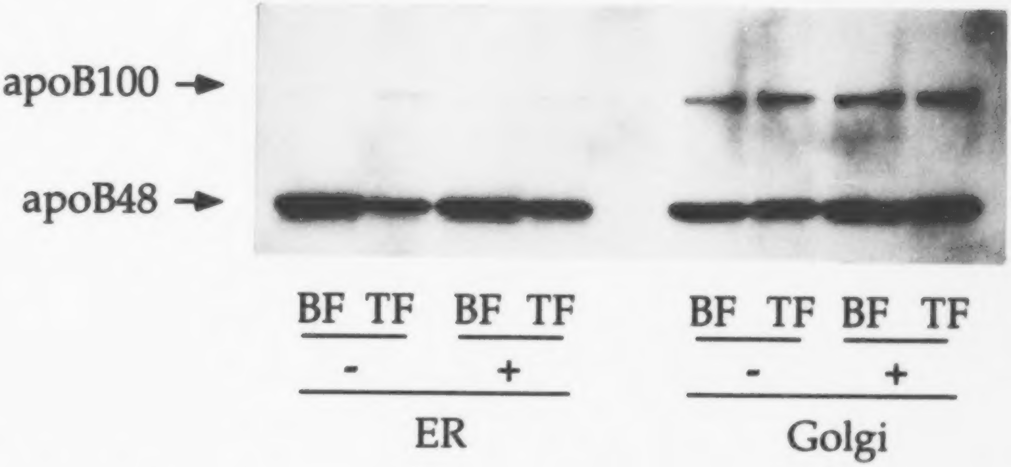


Figure 7. ApoB Levels in Lumina of ER and Golgi Fractions from CD and CS Livers and from CD and CS Plasma.

The luminal contents of ER and Golgi were prepared as described in "Materials and Methods". The amount of apoB was quantitated by an ELISA using a polyclonal rabbit anti-rat apoB antibody as a primary antibody. The values represent the means of 4 separate subcellular fractionations ± S.D. **, $p < 0.005$; * $p < 0.05$.

Figure 8. Density Distribution of ApoB in Luminal Contents of ER and Golgi Fractions from CS or CD Rat Livers.

Subcellular fractions were isolated from CD and CS rat livers, and the luminal contents were isolated after treatment of the ER (35 mg) and Golgi (8 mg) with sodium carbonate. The luminal contents were separated into two fractions (d< 1.01 g/ml, d≥ 1.01 g/ml) by density gradient ultracentrifugation. The d< 1.01 g/ml fraction corresponds to VLDL. Lipoproteins were concentrated on to Cab-O-Sil™ as described in Materials and Methods. Apoproteins were released from Cab-O-Sil™, after lipid extraction by treatment with lipoprotein sample buffer and separated by SDS-polyacrylamide gels, transferred to PVDF membrane, and incubated with rabbit anti-rat VLDL antibody, followed by peroxidase-conjugated anti-rabbit IgG. The proteins were detected by enhanced chemiluminescence assay. No choline in the rat diet is indicated by -, and + indicates that there was choline in the diet. BF, d≥ 1.01 g/ml; TF, d< 1.01 g/ml. The experiment was repeated 3 times with similar results.


apoB100
apoB48
BF
TF
BF
TF
BF
TF
BF
TF
-
+
-
+
ER
Golgi

increase in d< 1.01 g/ml apoB100 from both CD and CS Golgi lumina relative to ER lumina is not clear. There was less apoB48 and apoB100 in CD- relative to CS-Golgi lumina confirming the results discussed above that showed less apoB in CD-Golgi lumina.

3.4 Albumin Levels in ER and Golgi Lumina

As a control, albumin levels were examined in the lumina of the subcellular fractions using ELISA. As shown in Table III, albumin levels are not significantly different between CD and CS subcellular fractions examined or CD and CS plasma. This suggests that normal protein secretion is not impaired in CD, relative to CS conditions.

3.5 Translocation of ApoB Across the ER Membrane

One possibility for the defect in VLDL secretion caused by choline deficiency is that translocation of apoB into the ER lumen is impaired. When intact microsomes are prepared from liver homogenates or cultured hepatocytes, more than 50% of the apoB is associated with the membrane and is accessible to trypsin digestion (Davis *et al.*, 1990; Wilkinson *et al.*, 1990; Wilkinson *et al.*, 1992b). From the subcellular fractionation studies discussed above, there are no major differences in the number of apoB containing particles in the ER lumina between CD and CS rats, suggesting that translocation was not a factor. Also, since there is no effect on apoB synthesis caused by choline deficiency (Yao and Vance, 1988), the susceptibility of apoB on the ER membrane to trypsin would be expected to be similar between CD and CS samples. Therefore, intact microsomes were isolated from CD and CS hepatocytes which had been labeled to equilibrium (16 hours) with [^{3}H] leucine (20 μCi/dish), and incubated in the absence (control) or presence of TPCK-trypsin (60 μg/400 μg microsomal protein) for 30 min at 4°C. The

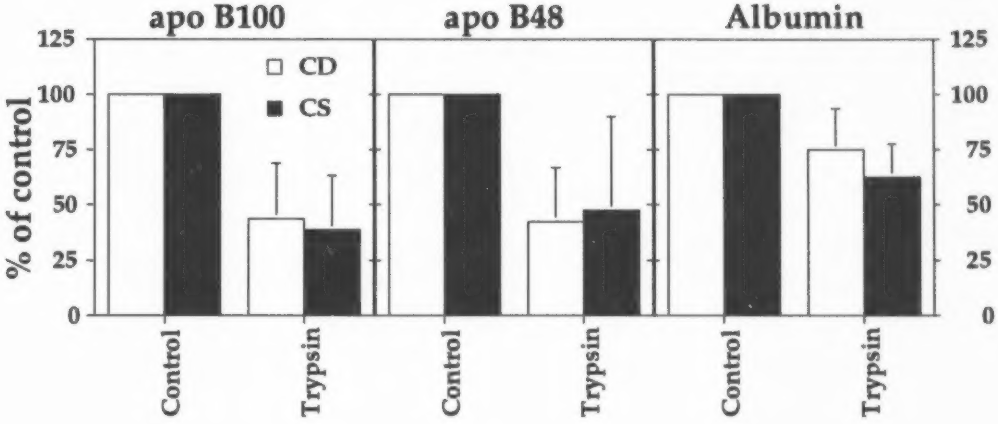


Figure 9. Accessibility of ApoB to Trypsin in Microsomes from CD and CS Hepatocytes.

Microsomes were isolated from CD and CS hepatocytes that had been labeled overnight (16 h) with [^{3}H] leucine (20 µCi/dish). The labeled microsomes were incubated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-trypsin (60 µg/400µg microsomal protein) or without (control) for 30 min at 4°C. The reaction was stopped by the addition of a protease inhibitor cocktail (soybean trypsin inhibitor, 1.25 mg/ml, aprotinin, 1 µg/ml, leupeptin, 50 µg/ml, and 1 µM phenylmethylsulfonylfluoride) plus addition of an equal volume of 2 X SDS-PAGE sample buffer, and immediately boiled for 5 min. The proteins were separated by 3-15% SDS-PAGE and stained with Coomassie Blue R250. Bands corresponding to apoB100, apoB48 and albumin were cut from the gel and dissolved overnight in 0.25 ml perchloric acid and 0.5 ml H_2O_2 (30%) at 60°C and radioactivity determined. The results are means of 3 separate experiments ± S.D.

reaction was stopped by the addition of a protease inhibitor mixture (1.25 mg/ml soybean trypsin inhibitor, 1 µg/ml aprotinin, 50 µg/ml leupeptin, and 1 µM PMSF) plus an equal volume of 2x SDS PAGE sample buffer and immediately boiled for 5 min. It is evident from Figure 9 that in intact microsomes from both CS and CD cultured hepatocytes, approximately 70% of apoB48 and apoB100 was digested with exogenously added trypsin. In contrast, only 25 to 35% of albumin, the mature form of which is largely found in the lumina of microsomes, was degraded during the incubation with trypsin. Incubations in which trypsin was added immediately followed by a cocktail that contained trypsin inhibitor, PMSF, leupeptin and aprotinin, gave the same results as the control samples that contained no trypsin. Thus, the luminal contents of the microsomal membranes were largely protected from trypsin digestion. Since the trypsin susceptibility of both forms of apoB was similar in CD and CS microsomes, these experiments suggest that choline deficiency does not impair the translocation of either apoB100 or apoB48 into the ER lumen. The experiment is consistent with the finding that apoB100 and apoB48 are found in the ER lumina of CD and CS livers at similar concentrations.

3.6 Intracellular Transport Inhibitor Studies

The work discussed above showed that equal amounts of apoB were present in ER lumina from CD and CS rat livers, but that the level of apoB in CD Golgi lumina was lower than in CS samples (Figure 7 and Table III). The next logical step was to determine the subcellular site of apoB degradation occurring in CD livers. We know that choline deficiency does not affect the amount of apoB synthesized but does affect the amount secreted (Yao and Vance, 1988; 1990). We used the intracellular transport inhibitors BFA and monensin to localize more precisely the site of this degradation.

3.6.1 Brefeldin A

Brefeldin A blocks the movement of proteins in the secretory pathway between the ER and the Golgi, and has been shown not to prevent ER based degradation of proteins (Klausner and Sitia, 1990). Moreover, the Golgi tends to redistribute into the ER (Klausner *et al.*, 1992; Lippincott-Schwartz *et al.*, 1990). If the degradation of VLDL from CD livers was occurring in the ER, BFA treatment should result in increased degradation of apoB in the ER. Consequently, less apoB would be present in CD hepatocytes when compared to similarly treated CS hepatocytes. However, if the CD specific degradation was in a post-ER compartment, BFA treatment should result in protection of apoB and the amount of apoB in CD hepatocytes would be equivalent to that found in CS hepatocytes also treated with BFA. To distinguish between ER and post-ER degradation, CD and CS hepatocytes were pulse labeled for 30 min with 100 μCi/dish of [^{35}S] cell labeling mixture. The cells were washed twice with phosphate buffered saline, and radioactivity was chased with medium containing 200 μM methionine and either 5 μg/ml BFA in dimethylsulfoxide or an equivalent amount of dimethylsulfoxide alone. The cells were maintained at 37°C for 90 min after which VLDL was immunoprecipitated from both the cells and medium. Since BFA has been shown to block protein synthesis (Fishman and Curran, 1992), it was only present during the chase. The amount of labeled apoB (either apoB48 or apoB100) was the same in the CD and CS cells (Figures 11 and 13). The total amount of label incorporated into apoB (cells + medium) was equivalent in CD and CS hepatocytes when secretion was blocked with BFA. This is in agreement with previous results showing that the synthesis rate of apoB is

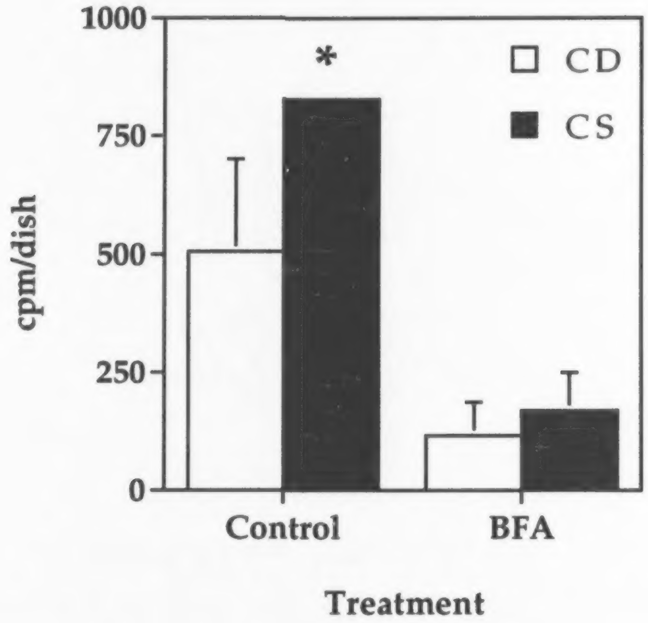


Figure 10. Effect of Brefeldin A on Secreted ApoB100.

CD and CS hepatocytes were pulse labeled for 30 min with [^{35}S] cell labeling mix (100 µCi/dish in 1 ml). The cells were washed twice with phosphate buffered saline and radioactivity was chased for a further 90 min in the presence or absence of 5 µg/ml BFA. The media was harvested and VLDL was immunoprecipitated. Proteins were separated on SDS polyacrylamide gels. The bands corresponding to apoB100 were excised from the gel and radioactivity was determined. Values are means ± S.E.M. of at least 3 separate experiments each done in duplicate. Values were normalized to account for varying amounts of label incorporated. * $p < 0.01$ CD vs. CS.

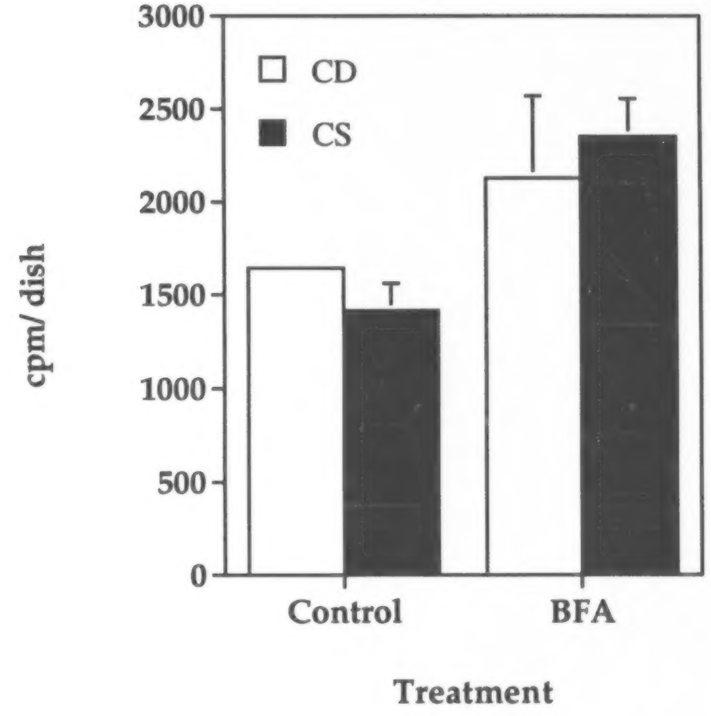


Figure 11. Effect of Brefeldin A on Intracellular ApoB100.

CD and CS hepatocytes were pulse labeled for 30 min with [^{35}S] cell labeling mix (100 µCi/dish in 1 ml). The cells were washed twice with phosphate buffered saline and radioactivity was chased for a further 90 min in the presence or absence of 5 µg/ml BFA. The cells were harvested and VLDL was immunoprecipitated. Proteins were separated on SDS polyacrylamide gels. The bands corresponding to apoB100 were excised from the gel and radioactivity was determined. Values are means ± S.E.M. of at least 3 separate experiments each done in duplicate. Values were normalized to account for varying amounts of label incorporated.

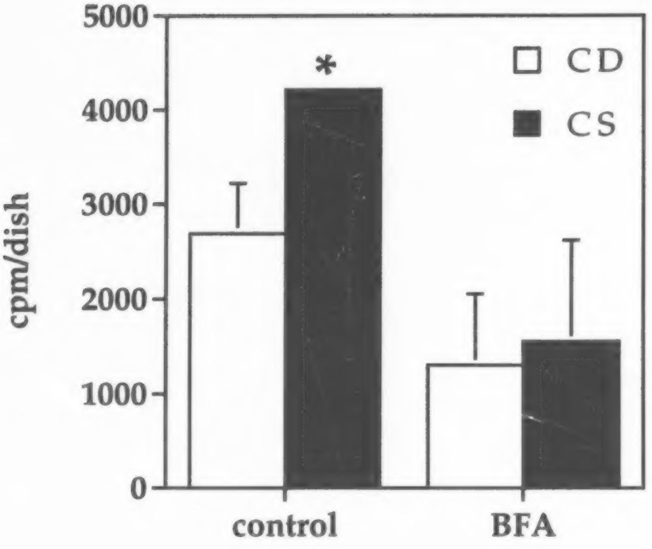


Figure 12. Effect of Brefeldin A on Secreted ApoB48.

CD and CS hepatocytes were treated as in the legend for Figure 10 except the bands corresponding to apoB48 were excised from the gel. Data shows radioactivity in apoB48 secreted into the medium during the chase period. Values are means ± S.E.M. of at least 3 separate experiments each done in duplicate. * $p< 0.01$ CD vs. CS.

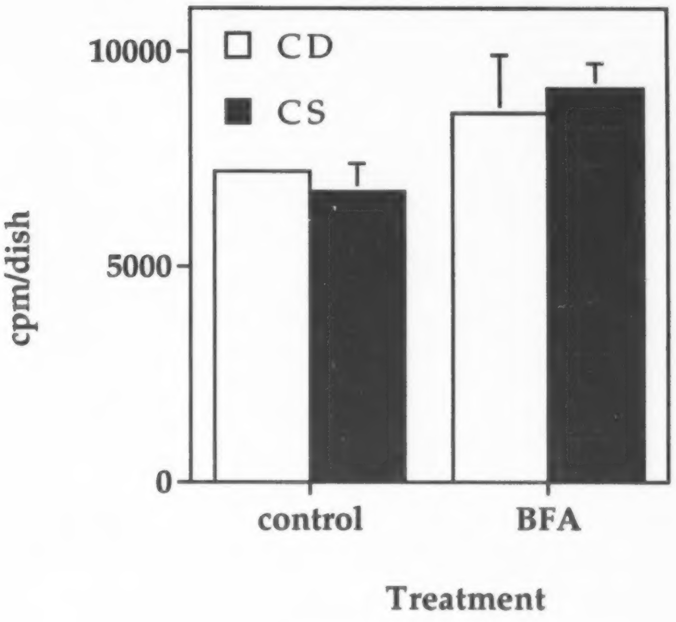


Figure 13. Effect of Brefeldin A on Intracellular ApoB48.

CD and CS hepatocytes were treated as in the legend for Figure 11 except the bands corresponding to apoB48 were excised from the gel. Data show radioactivity in apoB48 remaining intracellularly during the chase. Values are means ± S.E.M. of at least 3 separate experiments each done in duplicate. Values were normalized to account for varying amounts of label incorporated.

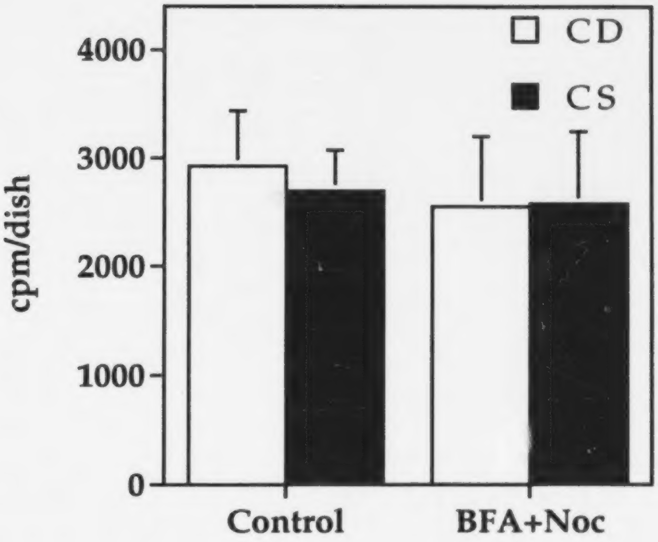


Figure 14 Effect of Brefeldin A and Nocodazole on Intracellular ApoB100

CD and CS hepatocytes were pulse labeled for 30 min with [^{35}S] cell labeling mix (100 µCi/dish in 1 ml). The cells were washed twice with phosphate buffered saline and radioactivity was chased for a further 90 min in the presence or absence of 5 µg/ml BFA and 10 µg/ml nocodazole. The cells were harvested and VLDL was immunoprecipitated. Proteins were separated on SDS polyacrylamide gels. The bands corresponding to apoB100 were excised from the gel and radioactivity was determined. Values are means ± S.E.M. of at least 3 separate experiments each done in duplicate. Values were normalized to account for varying amounts of label incorporated.

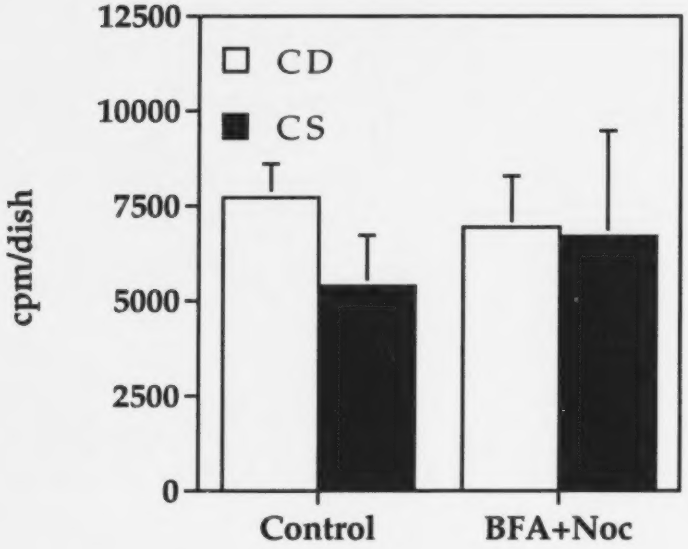


Figure 15 Effect of BrefeldinA and Nocodazole on Intracellular ApoB48

CD and CS hepatocytes were treated as in the legend for Figure 14 except the bands corresponding to apoB48 were excised from the gel. Data show radioactivity in apoB48 remaining intracellularly during the chase. Values are means ± S.E.M. of 3 separate experiments, each done in duplicate. Values were normalized to account for varying amounts of label incorporated.

similar under CD and CS conditions (Yao and Vance, 1988). However, less apoB48 and apoB100 were secreted into the medium from CD hepatocytes (Figures 10 and 12). BFA blocked secretion of both apoB100 and apoB48 into the medium (Figures 10 and 12). Secretion of apoB100, apoB48 and albumin was also inhibited when nocodazole (10 μg/ml) along with BFA was included in the chase medium (data not shown). Nocodazole, a microtubule disrupting agent, prevents the retrograde transport of Golgi membranes to the ER (Lippincott-Schwartz *et al.*, 1990). In the cells, addition of BFA plus nocodazole did not further protect apoB100 or apoB48 from degradation (Figures 14 and 15). This suggests that, in experiments with BFA, retrograde transport of the Golgi to the ER is not affecting apoB degradation. The results shown suggest that the increased degradation of apoB observed in CD, compared to CS, hepatocytes occurs in a post-ER compartment.

3.6.2 Monensin

Monensin is an ionophore that disrupts Na+ and H+ gradients. It has numerous cellular effects including inhibition of protein secretion and interference with Golgi function (Mollenhauer *et al.*, 1990). Most of monensin's effects are specifically directed at the *trans*-Golgi. However, several studies have found in rat hepatocytes that monensin prevents processing of N-linked carbohydrate chains and interferes with transport from the ER (reviewed in, Mollenhauer *et al.*, 1990). Monensin has previously been shown to block VLDL secretion (Melin *et al.*, 1984; Rustan *et al.*, 1985) at the level of the Golgi (Rustan *et al.*, 1987)

In this series of experiments, CD and CS hepatocytes were pulse labeled for 30 min with 100 μCi/dish of [^{35}S] cell labeling mixture. The cells were washed twice with phosphate buffered saline, and radioactivity was chased with medium containing 200 μM methionine and either 10 μM

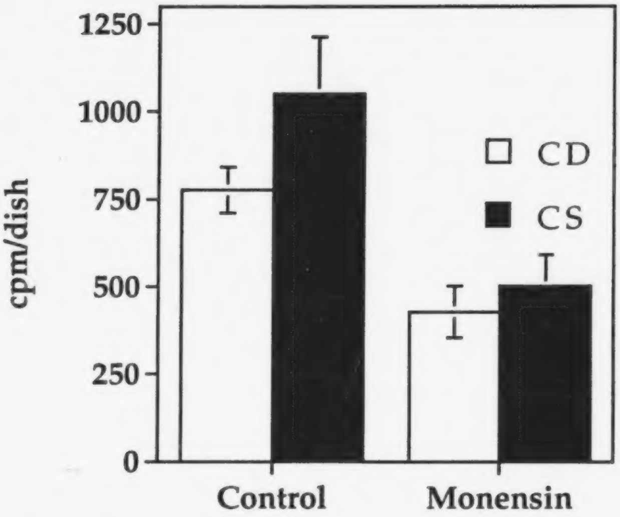


Figure 16. Effect of Monensin on Secreted ApoB100.

CD and CS hepatocytes were pulse labeled for 30 min with [^{35}S] cell labeling mix (100 μCi/dish in 1 ml). The cells were washed twice with phosphate buffered saline and radioactivity was chased for a further 90 min in the presence or absence of 10 μM monensin. The media was harvested and VLDL was immunoprecipitated. Proteins were separated on SDS polyacrylamide gels. The bands corresponding to apoB100 were excised from the gel and radioactivity was determined. Values are means ± S.E.M. of at least 3 separate experiments each done in duplicate. Values were normalized to account for varying amounts of label incorporated. * $p < 0.01$ CD vs. CS.

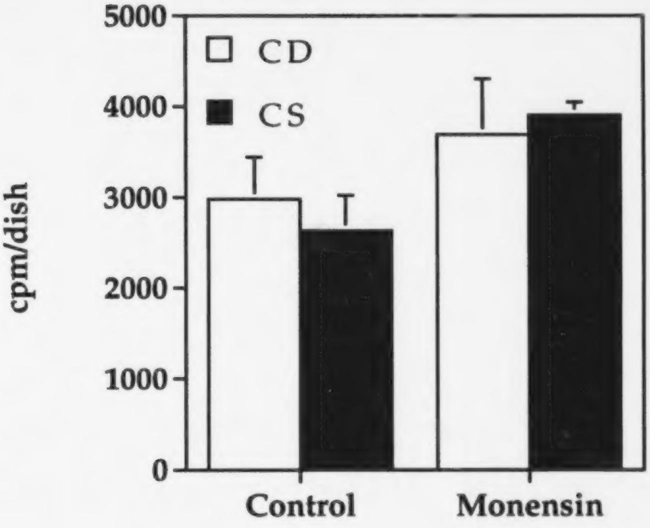


Figure 17. Effect of Monensin on Intracellular ApoB100.

CD and CS hepatocytes were pulse labeled for 30 min with [^{35}S] cell labeling mix (100 µCi/dish in 1 ml). The cells were washed twice with phosphate buffered saline and radioactivity was chased for a further 90 min in the presence or absence of 10 µM monensin. The cells were harvested and VLDL was immunoprecipitated. Proteins were separated on SDS polyacrylamide gels. The bands corresponding to apoB100 were excised from the gel and radioactivity was determined. Values are means ± S.E.M. of at least 3 separate experiments each done in duplicate. Values were normalized to account for varying amounts of label incorporated.

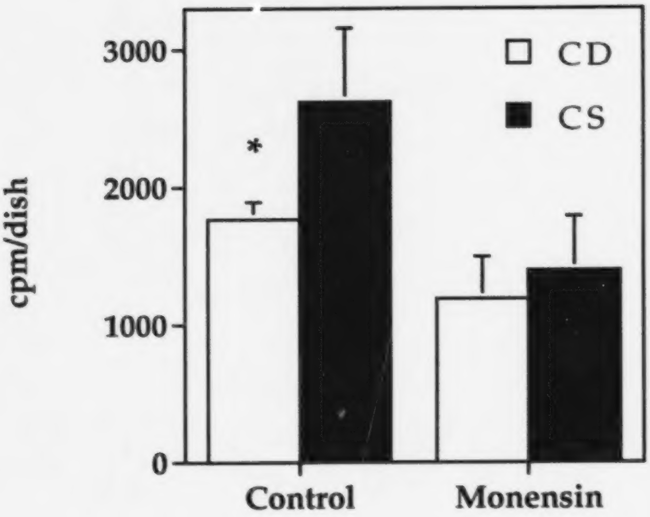


Figure 18. Effect of Monensin on Secreted ApoB48.

CD and CS hepatocytes were treated as in the legend for Figure 16, except bands corresponding to apoB48 were analysed. Data shows radioactivity in apoB48 secreted into the medium during the chase period. Values are means ± S.E.M. of at least 3 separate experiments each done in duplicate. * $p < 0.01$ CD vs. CS.

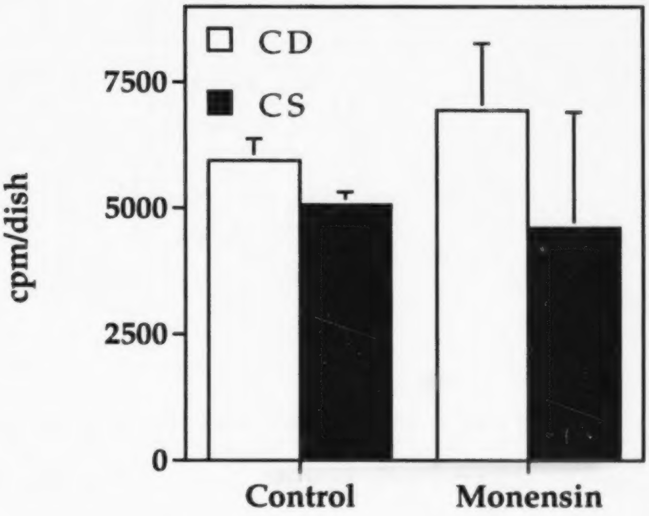


Figure 19. Effect of Monensin on Intracellular ApoB48.

CD and CS hepatocytes were treated as in the legend for Figure 17, except bands corresponding to apoB48 were analysed. Data show radioactivity in apoB48 remaining intracellularly during the chase. Values are means ± S.E.M. of at least 3 separate experiments each done in duplicate, normalized to account for varying amounts of label incorporated.

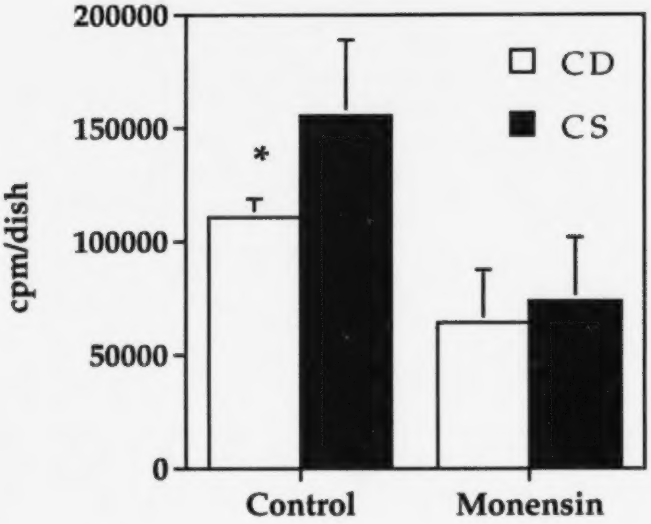


Figure 20. Effect of Monensin on Secreted Albumin.

CD and CS hepatocytes were treated as in the legend for Figure 16 except albumin was immunoprecipitated. Data shows radioactivity in albumin secreted into the medium during the chase period. Values are means ± S.E.M. of at least 3 separate experiments each done in duplicate, normalized to account for varying amounts of label incorporated. * $p < 0.01$ CD vs. CS.

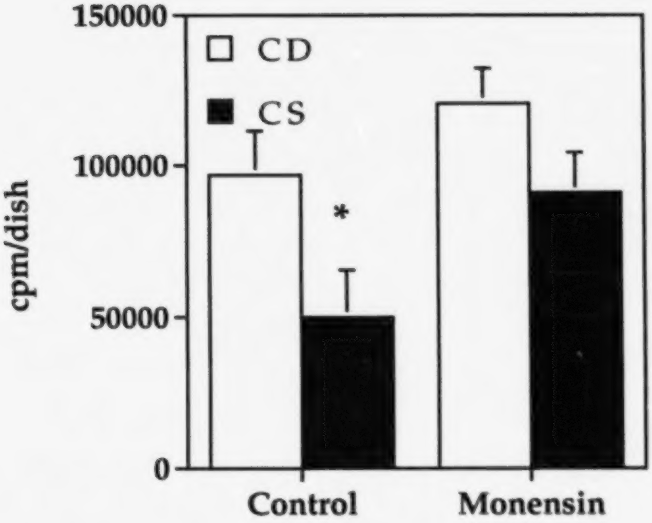


Figure 21. Effect of Monensin on Intracellular Albumin.

CD and CS hepatocytes were treated as in the legend for Figure 17 except albumin was immunoprecipitated. Data show radioactivity in albumin remaining intracellularly during the chase. Values are means ± S.E.M. of at least 3 separate experiments each done in duplicate, normalized to account for varying amounts of label incorporated. * $p < 0.01$ CD vs. CS.

monensin in ethanol (0.01% v/v final concentration) or an equivalent amount of ethanol alone. The cells were maintained at 37°C for 90 min after which VLDL was immunoprecipitated from both the cells and medium. Monensin was only present during the chase. If apoB degradation was occurring in the *trans*-Golgi, i.e., after the monensin blockage of secretion, the levels of apoB should be equivalent between CD and CS hepatocytes treated with monensin. However, if apoB degradation was occurring prior to the *trans*-Golgi, there should be less apoB present in CD samples treated with monensin. The results are compounded by the inefficiency of the secretion blockage caused by monensin treatment as observed in Figures 16, 18 and 20. The amounts of labeled apoB (either apoB100 or apoB48) were not statistically different in CD and CS cells (Figures 17 and 19). The total amount of label incorporated into apoB (cells + medium) was equivalent in CD and CS hepatocytes when secretion was blocked with monensin. This is in agreement with previous results showing that the synthesis rate of apoB is similar under CD and CS conditions (Yao and Vance, 1988). However, less apoB48 and apoB100 were secreted into the medium from CD than CS hepatocytes (Figures 16 and 18). The blockage of secretion caused by monensin is not absolute as a relatively large amount of label is secreted in the presence of monensin (Figures 16, 18 and 20). This may be a consequence of the time (approximately 15 min) required for monensin to block secretion (Rustan *et al.*, 1987). Monensin treatment of CD and CS hepatocytes caused a small accumulation of apoB100 (Figure 17). However, apoB48 and albumin did not accumulate in cells in the presence of monensin (Figures 19 and 21). The studies using monensin are inconclusive due to multiple sites of monensin interaction with cells as previously observed (Mollenhauer *et al.*,

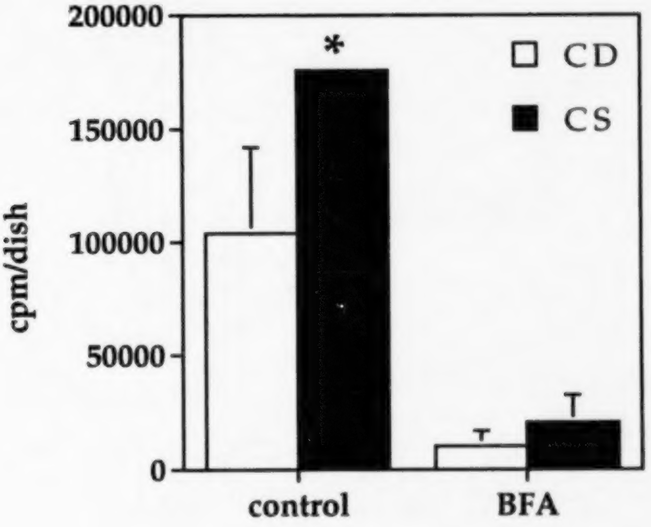


Figure 22. Effect of Brefeldin A on Secreted Albumin.

CD and CS hepatocytes were treated as in the legend for Figure 10 except albumin was immunoprecipitated. Data show radioactivity associated with albumin secreted into the medium during the chase period. Values are means ± S.E.M. of at least 3 separate experiments each done in duplicate, normalized to account for varying amounts of label incorporated. * $p < 0.01$ CD vs. CS.

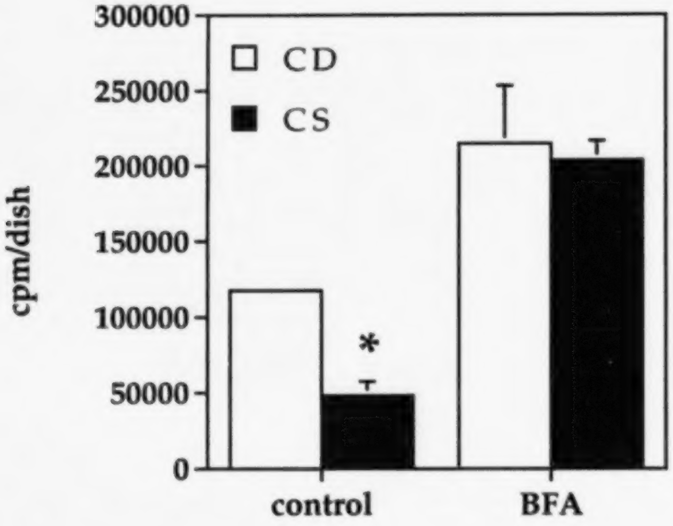


Figure 23. Effect of Brefeldin A on Intracellular Albumin.

CD and CS hepatocytes were treated as in the legend for Figure 11 except albumin was immunoprecipitated. Data show radioactivity in albumin remaining intracellularly during the chase. Values are means ± S.E.M. of at least 3 separate experiments each done in duplicate, normalized to account for varying amounts of label incorporated. * $p < 0.01$ CD vs. CS.

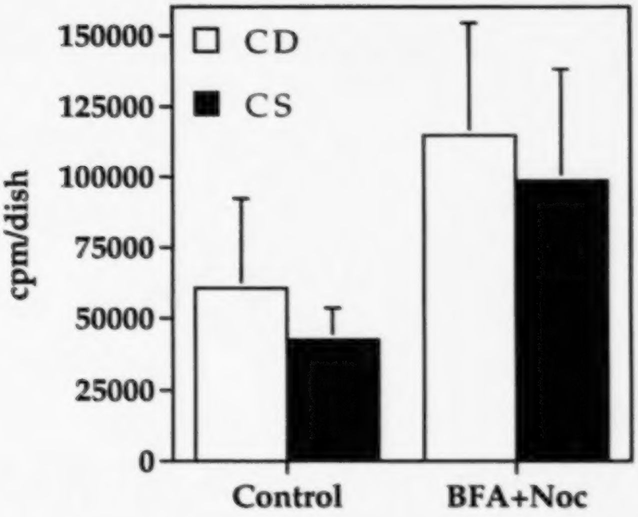


Figure 24. Effect of Brefeldin A and Nocodazole on Intracellular Albumin.

CD and CS hepatocytes were treated as in the legend for Figure 14 except albumin was immunoprecipitated. Data show radioactivity in albumin remaining intracellularly during the chase. Values are means ± S.E.M. of at least 3 separate experiments each done in duplicate. Values were normalized to account for varying amounts of label incorporated.

1990). However, the results do not contradict the studies with BFA that suggest a post-ER degradation of apoB.

3.7 Protein Secretion Rate Studies

3.7.1 Albumin

During the experiments with BFA, we analyzed the secretion of albumin as a typical secretory protein. The amount of radiolabeled albumin secreted from CD hepatocytes was 40% lower than from CS hepatocytes (Figure 22). In the CD samples there was a corresponding increase (245% of CS) of intracellular albumin (Figure 23). The total amount of radiolabeled albumin synthesized did not differ between CD and CS cells (221,460 vs. 223,630 cpm/dish, respectively). When protein secretion was blocked with BFA, no degradation of the labeled albumin was observed, and the amount of intracellular labeled albumin was equivalent in CD and CS cells. An inhibition of albumin secretion from CD hepatocytes was unexpected since, in plasma, the levels of albumin/mg protein are equivalent in CD compared to CS rats. Nocodazole experiments with albumin showed similar results (Figure 24) as experiments with BFA alone for both CD and CS hepatocytes.

3.7.2 Bulk Proteins

We then examined the rate of bulk protein secretion by pulse-labeling the cells for 30 min with [^{3}H]-leucine and chasing the labeled proteins into the medium for up to four h. Labeled proteins in the cells and medium were quantitated after TCA precipitation (Figures 25 and 26). Figure 25 shows that the rate of protein secretion from CD cells was approximately 80% of the CS value. The initial rates of protein secretion ($\leq$ 2 h) were calculated to be 50,670 dpm/mg/h from CD cells and 62,800 dpm/mg/h from CS cells. The rate of loss of label from the cellular proteins was indistinguishable between

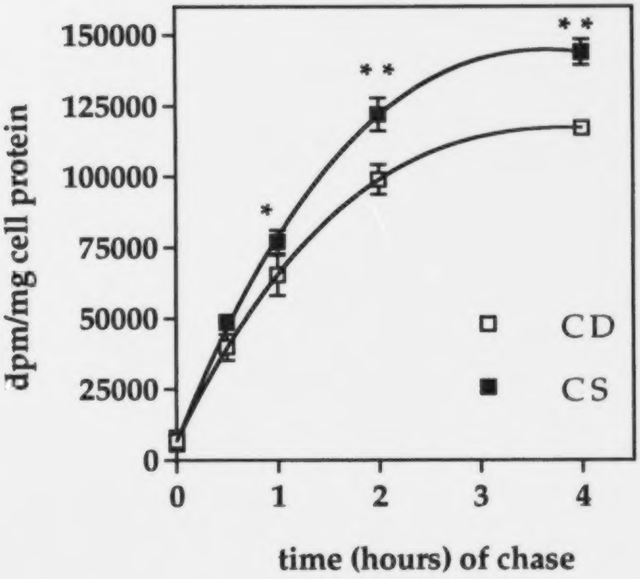


Figure 25. Secretion of Bulk Proteins from CD and CS Hepatocytes.

CD and CS hepatocytes were pulse labeled for 30 min with 25 µCi/dish of [^{3}H]-leucine, washed twice with 2 ml of phosphate buffered saline and radioactivity chased for up to 4 h in medium containing unlabeled leucine. Labeled proteins were precipitated from medium with 10% TCA. The results are expressed as dpm/mg cellular protein. They are the means ± standard deviation of 3 separate experiments each performed in duplicate. * $p< 0.05$, **$p< 0.01$ CD vs. CS.

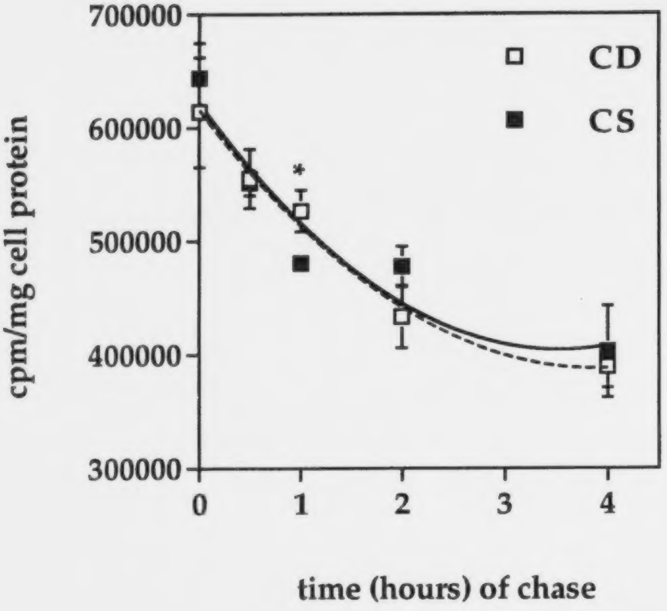


Figure 26. Release of Bulk Proteins from CD and CS Hepatocytes

Hepatocytes were treated as in Figure 25, except labelled proteins were precipitated from cells. The results are expressed as dpm/mg cellular protein. The data are the means ± S.D. of 3 separate experiments each performed in duplicate. * $p< 0.05$ CD vs. CS.

CD and CS cells (Figure 26). The amount of label incorporated into cellular protein at the end of the pulse period (t=0 chase) was equivalent in CD vs. CS hepatocytes (613,960 ± 49,000 vs. 643,680 ± 31,000 dpm/mg respectively). These data suggest that choline deficiency slightly lowers the rate of bulk protein secretion, although apoB secretion is affected to a greater degree (Yao and Vance, 1988).

3.8 Characterization of Nascent VLDL from ER and Golgi Lumina

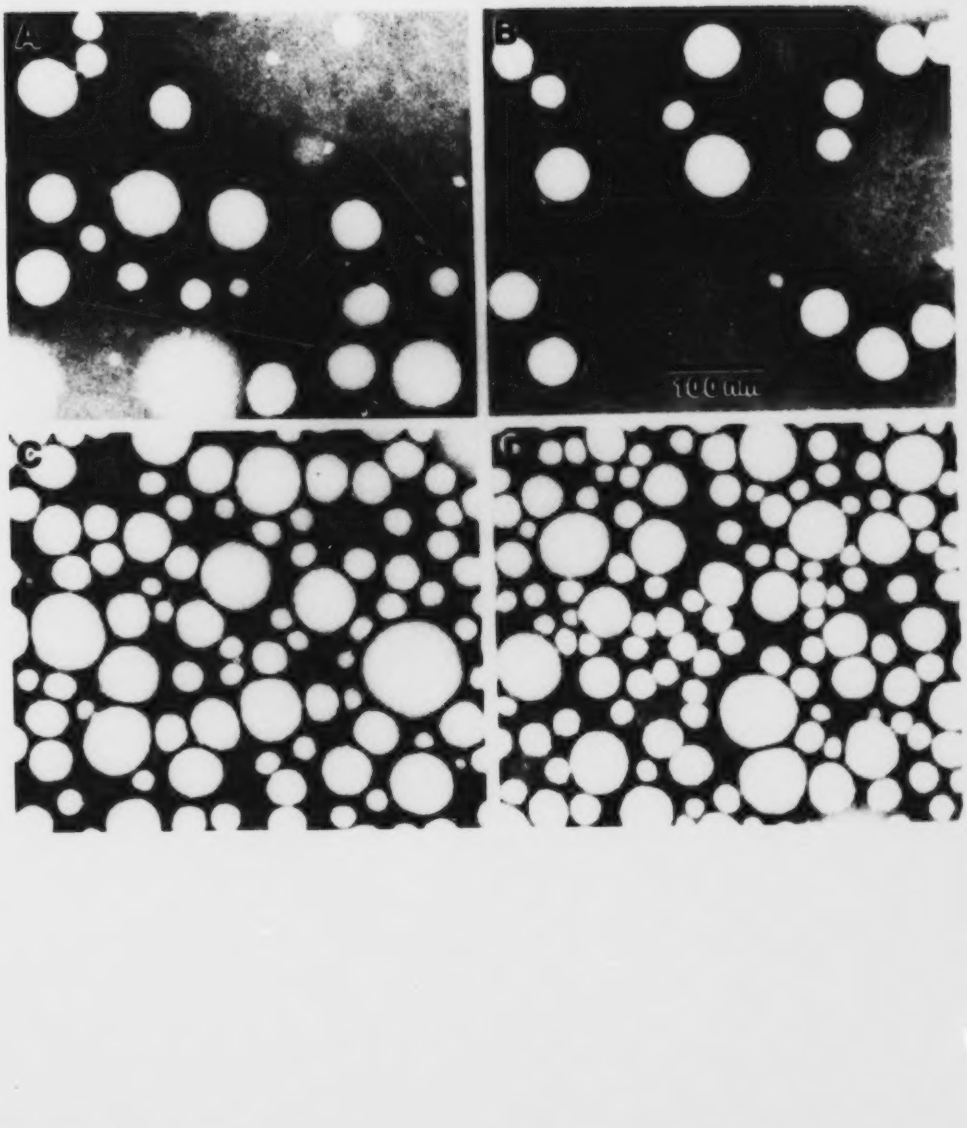
To determine if the physical properties of nascent VLDL from CD rat liver were responsible for targeting the particles for degradation, we examined the size and lipid composition of the particles isolated from the lumina of the ER and Golgi. These were compared to particles isolated from the plasma of CD and CS rats.

3.8.1 Size of Nacsent VLDL

The luminal contents of Golgi from CD and CS livers were isolated after sequential flotation ultracentrifugation. The fraction with a density <1.006 g/ml (VLDL) was used for negative staining electron microscopy (Figure 27 A, B). There appeared to be more particles with larger diameters in the CD compared to CS samples. The diameters of the visualized particles were measured and plotted into histograms (Figure 28). The mean particle size in the Golgi fraction was greater in CD than in CS liver, 46.1 ± 13.1 nm versus 40.0 ± 13.7 nm, respectively ($P < 0.001$, non-paired t-test, n=250). This difference in the average value was mostly due to a relative abundance of particles with a diameter between 50 and 80 nm in the CD fractions. The VLDL from the ER lumina was not examined by this method.

Figure 27. Electron Microscopy of d< 1.006 g/ml Fraction of Golgi Lumina and Plasma VLDL from CD and CS Rats

Plasma VLDL and the d<1.006 g/ml fraction of Golgi lumina were isolated by sequential flotation centrifugation and analyzed by negative staining electron microscopy as described in Materials and Methods. Panel A, CD Golgi; Panel B, CS Golgi; Panel C, CD plasma; Panel D, CS plasma.


A
B
100 nm
C
D

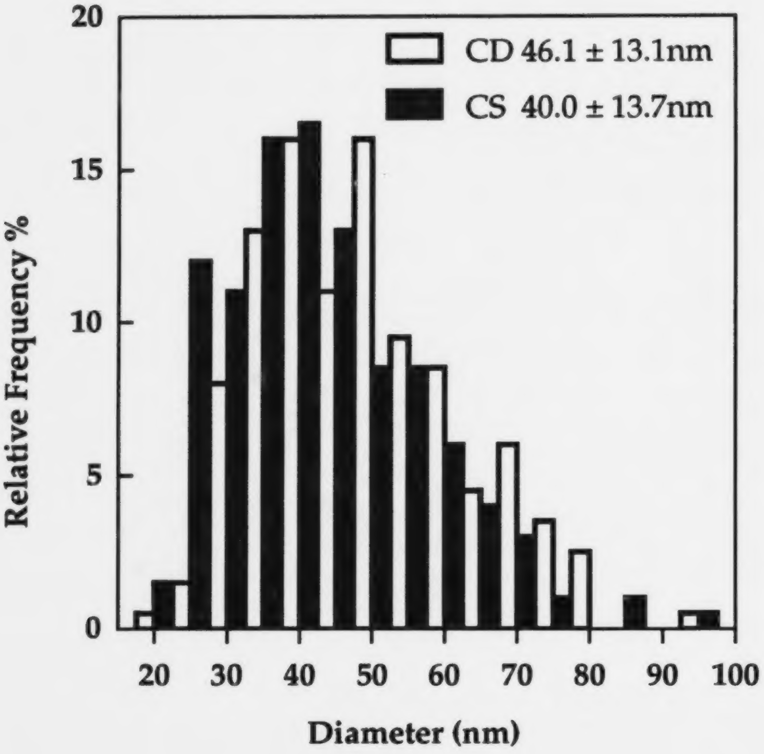


Figure 28. Histograms of the d<1.006 g/ml Fraction Isolated from the Golgi Lumina from CD and CS Rat Liver.

Measurements were made of the diameters of 250 particles from CD and CS Golgi. Examples of the corresponding images that were used for diameter measurements are shown in Figure 27.

3.8.2 Lipid Analysis

We wished to gain additional information on the signal that might be involved in targeting the VLDL from CD liver for degradation. One hypothesis is that the CD particles are abnormal in structure and, therefore, are specifically targeted to degradation. We therefore investigated whether the phospholipid composition of nascent VLDL was different in CD and CS rat livers. Nascent lipoproteins were isolated from the lumina of ER and Golgi from CD and CS rat livers by density gradient ultracentrifugation. The lumina were separated into two fractions; one of d< 1.01 g/ml (equivalent to VLDL density) and the other of d≥ 1.01 g/ml. Further fractionation into other lipoprotein classes such as HDL or LDL was not possible due to lack of material. The lipoproteins were concentrated onto Cab-O-Sil™, lipids extracted and separated by thin-layer chromatography, and the masses of PC, PE, and TG were measured (Table IV). In all CD fractions (except ER d< 1.01 g/ml) examined, the amount of PC was decreased relative to that in the corresponding CS fractions. In each case there was no change in the level of PE. This leads to a lower PC/PE ratio in CD, compared to CS fractions (Figure 29). When the amounts of total phospholipid in the lumina were expressed as nmol/µg apoB found in the lumina of the ER and Golgi as described in section 3.2, the levels of PC/µg apoB were lower in samples from CD liver compared to CS liver (Table V). The levels of PE per apoB (Table V) did not change in ER lumina (6.0 vs. 6.1 nmol/µg apoB, CD vs. CS, respectively), but were higher in Golgi lumina from CD, than from CS liver (5.4 vs. 4.3 nmol/µg apoB, respectively). This observation supports a change in lipoprotein phospholipid composition, but does not specifically examine the particles in the d< 1.01 g/ml fraction, and does not distinguish between

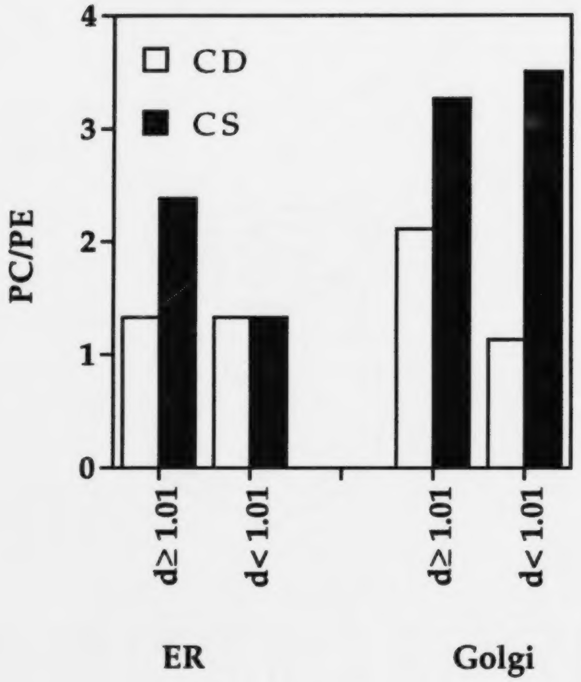


Figure 29. PC/PE Ratios of Luminal Contents from ER and Golgi

The luminal contents of ER and Golgi from CD and CS rat liver was prepared as described in Table IV. The results are expressed as the ratio of PC/PE in the each fraction.

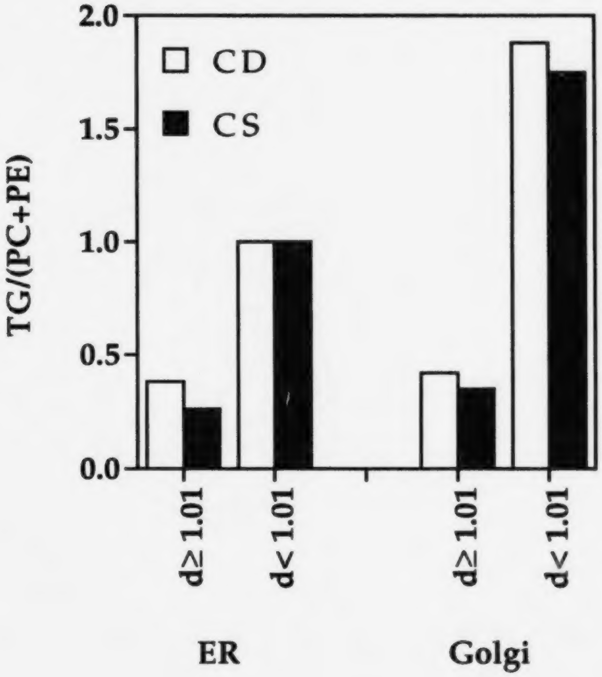


Figure 30. Ratio of TG/(PC+PE) of Luminal Contents from ER and Golgi

The luminal contents of ER and Golgi from CD and CS rat liver was prepared as described in Table IV. The results are expressed as the ratio of TG/(PC+PE) in each fraction.

Table IV

Lipid Composition of Luminal Contents from ER and Golgi

Luminal lipoproteins of subcellular fractions were isolated as described in Figure 8. The luminal contents were separated into two fractions ($d< 1.01$ g/ml, $d\geq 1.01$ g/ml) by density gradient ultracentrifugation. The $d< 1.01$ g/ml fraction corresponds to VLDL. Lipoproteins were concentrated on to Cab-O-Sil™ and lipids were extracted from each fraction, separated by thin-layer chromatography and quantitated. Values are mean nmol/mg organelle protein ± S.E.M. of at least 6 separate experiments.

Sample	Diet	PC	PE	TG
ER	CD	1.2 ± 0.3[b]	0.9 ± 0.2	0.8 ± 0.2
$d\geq 1.01$	CS	1.9 ± 0.5	0.8 ± 0.2	0.7 ± 0.1
ER	CD	0.4 ± 0.3	0.3 ± 0.3	0.7 ± 0.2
$d<1.01$	CS	0.4 ± 0.3	0.3 ± 0.3	0.7 ± 0.3
Golgi	CD	5.7 ± 3.4	2.7 ± 1.5	3.6 ± 1.1
$d\geq 1.01$	CS	12.4 ± 7.5	3.8 ± 1.6	5.7 ± 2.0
Golgi	CD	1.8 ± 0.8[b]	1.6 ± 0.7	6.4 ± 1.4[a]
$d < 1.01$	CS	6.3 ± 2.6	1.8 ± 0.7	14.2 ± 5.9

[a] $p< 0.05$, [b] $p< 0.01$ CD vs. CS

Table V

Lipid per ApoB in ER and Golgi Lumina

The sum of lipid species in top and bottom fractions of ER and Golgi lumina (Table IV) were expressed relative to the amount of apoB present in the lumina of the corresponding fraction (Figure 7). The values are expressed as nmol lipid/µg apoB.

	CD			CS		
luminal contents of	PC	PE	TG	PC	PE	TG
ER	8.0	6.0	7.5	12.7	6.1	7.8
Golgi	9.4	5.4	12.5	14.4	4.3	15.0

apoB48 and apoB100 containing particles. Minor phospholipids (phosphatidylinositol, phosphatidylserine, sphingomyelin and lysoPC) were not quantitated due to the lack of material. In the higher density fractions from both ER and Golgi contents, no significant differences in the amount of TG were observed between CD and CS samples. However, in the VLDL fraction from Golgi lumina, the levels of TG in the CD fractions are significantly lower than that of the CS samples (Table IV). This corresponds to a decrease in the number of VLDL particles present in CD relative to CS Golgi as shown in Figures 6 and 7. The ratios of TG to the sum of PC and PE were not significantly different between CD and CS samples (Figure 30). However, in the Golgi $d < 1.01$ g/ml fraction, the CD particles appeared to have a slightly higher TG/(PC + PE) ratio than corresponding CS particles (1.88 vs. 1.75). These data suggest the CD particles are slightly larger. However, the data did not prove to be significantly different by t-test. The amount of TG per apoB (Table V) appeared to be decreased in the Golgi lumina of CD, relative to CS subfractions (7.5 vs. 7.8 and 12.5 vs. 15.0 nmol/µg apoB for ER and Golgi, respectively). These calculations, however, do not take into account the relatively large proportion of the apoB in the lumina that is lipid poor (found in the $d \geq 1.01$ g/ml fraction), and therefore do not accurately represent nascent VLDL TG/apoB ratios. A slightly higher TG/phospholipid ratio in CD-derived nascent VLDL agrees well with the electron microscopy measurements (Figures 27 and 28) that showed the nascent VLDL from CD Golgi lumina were larger than CS-derived VLDL (46.1 ± 13.1 nm vs. 40.0 ± 13.7 nm, $p < 0.001$).

3.9 Composition of Plasma Lipoproteins

3.9.1 Size Analysis

Using the same methods described in section 3.8.1, we determined the average particle size of the VLDL fraction in plasma from CD and CS rats (Figure 24 C, D and Figure 31). It was previously shown that in CD rats, the amounts of VLDL-apoB and TG in the plasma are reduced by 60-80% compared to those in controls (Yao and Vance, 1990). Interestingly, the VLDL particles that are present in CD plasma had a larger average diameter than did plasma VLDL from CS rats, 44.6 ± 15.1 nm versus 37.9 ± 12.4 nm, respectively ($P < 0.001$, non-paired t-test, n=500). As was observed in the d<1.006 g/ml fraction from Golgi of CD rats, the histogram of plasma VLDL showed a relatively increased abundance of particles with a diameter larger than 50 nm, when compared to the plasma VLDL from CS rats (Figure 31). Three different isolations of plasma VLDL from CD and CS rats were performed, and the mean particle sizes found in each were 44.2 ± 16.3 (n=200), 43.7 ± 16.8 (n=150), and 45.6 ± 12.7 (n=150) for VLDL from CD rats, and 38.7 ± 12.5 (n=200), 37.9 ± 10.1 (n=150), and 36.9 ± 14.3 (n=150) nm for VLDL from CS rats.

3.9.2 Lipid Analysis

For comparison of nascent lipoproteins from the ER and Golgi lumina with their secreted counterparts, lipoprotein subfractions of three densities; d> 1.21 g/ml (BF), d= 1.21 g/ml (HDL), and d≤ 1.06 g/ml (VLDL + LDL) were prepared from CD and CS plasma. LDL was not analyzed separately due to the small amounts of LDL in rat plasma. Each fraction was washed by re-spinning the isolated lipoproteins through a potassium bromide solution of equivalent density to remove any contaminating proteins. Lipids were extracted after absorption of lipoproteins to Cab-O-Sil™ (Vance *et al.*, 1984)

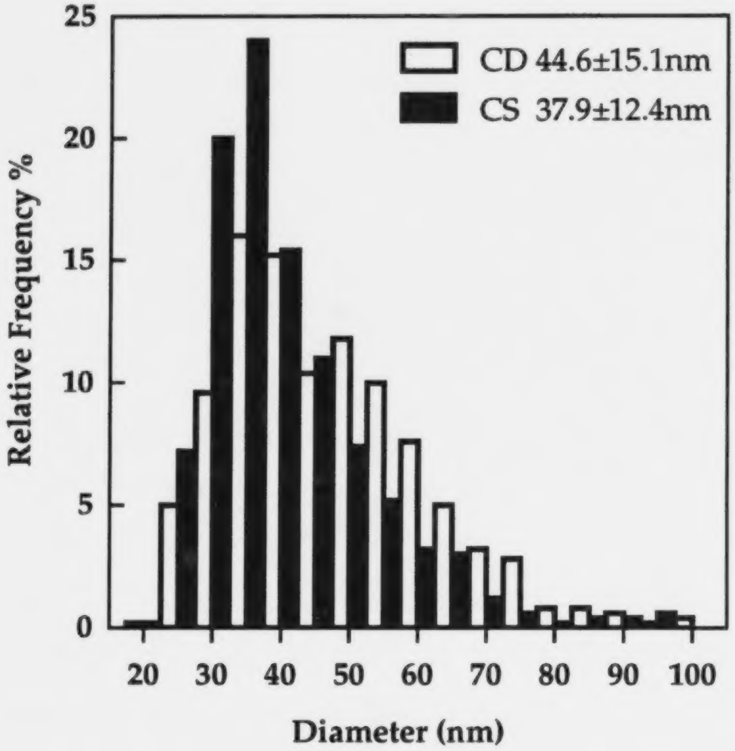


Figure 31. Histograms of Plasma VLDL Diameters Isolated from CD and CS Rats.

Measurements were made of the diameters of 500 particles from CD and CS plasma. Examples of the corresponding images that were used for diameter measurements are shown in Figure 27.

Table VI

Lipid Composition of Plasma Lipoproteins from CD and CS Rats

Plasma lipoproteins were isolated from CD or CS plasma (1.2 ml starting material) by sequential gradient density ultracentrifugation. The lipids were extracted, and the masses of PC, PE and TG were measured. The results are mean nmol/ml of plasma ± S.E.M of at least 3 separate experiments.

Fraction	Diet	PC	PE	TG
BF	CD	90.3 ± 25.2	14.5 ± 1.6	14.5 ± 4.5
d ≥ 1.21	CS	106.1 ± 21.9	13.7 ± 1.7	15.5 ± 6.0
HDL	CD	313.4 ± 36.6	18.9 ± 3.4	18.1 ± 5.1
d= 1.21	CS	367.1 ± 55.7	17.9 ± 1.0	15.4 ± 1.5
LDL+VLDL	CD	139.5 ± 17.1[a]	22.3 ± 4.9	301.8 ± 75.6
d≤ 1.06	CS	322.6 ± 31.0	25.8 ± 2.6	354.0 ± 66.1

[a] $p<0.01$ CD vs. CS

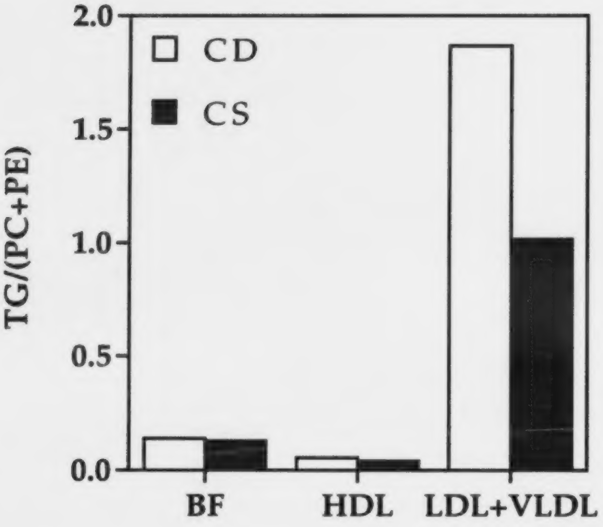


Figure 32. Ratio of TG/(PC+PE) of Plasma Lipoproteins from CD and CS Rats
Plasma lipoproteins were isolated as described in Table VI. The results are expressed as the ratio of TG/(PC+PE) in each fraction.

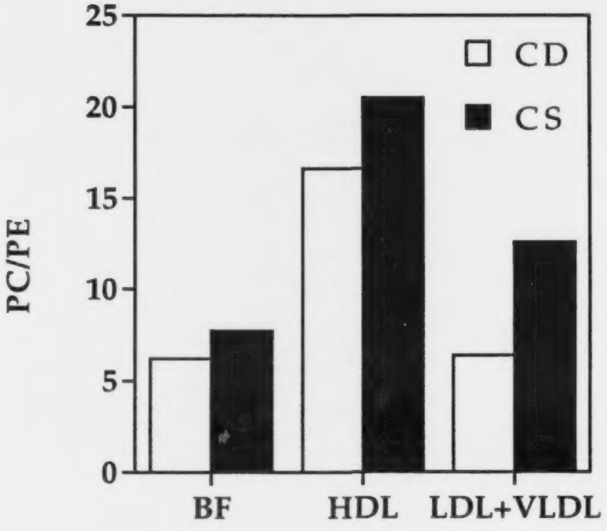


Figure 33. PC/PE Ratios of Plasma Lipoproteins from the Plasma of CD and CS Rats

Plasma lipoproteins were isolated as described in Table VI. The results are expressed as the ratio of PC/PE in each fraction.

and separated by thin-layer chromatography. The mass of PC, PE and TG was assayed from each fraction (Table VI). The d≤ 1.06 g/ml fraction from CD plasma has a higher TG/(PC + PE) ratio (Figure 32) than does CS plasma (1.86 versus 1.02, respectively), suggesting that the CD particles are slightly larger than the CS particles. This finding agrees well with the electron microscopy measurements that showed VLDL from CD plasma were slightly larger than corresponding particles isolated from CS plasma. Also, the ratio of PC/PE (Figure 33) was significantly lower in the CD than in the CS samples (6.4 vs. 12.6, respectively). The relative amount of TG compared to PC and PE was lower than expected for the LDL + VLDL fraction, suggesting that the preparations may have contained significant amounts of LDL.

3.9.3 Susceptibility of ApoB on Plasma VLDL to Proteases

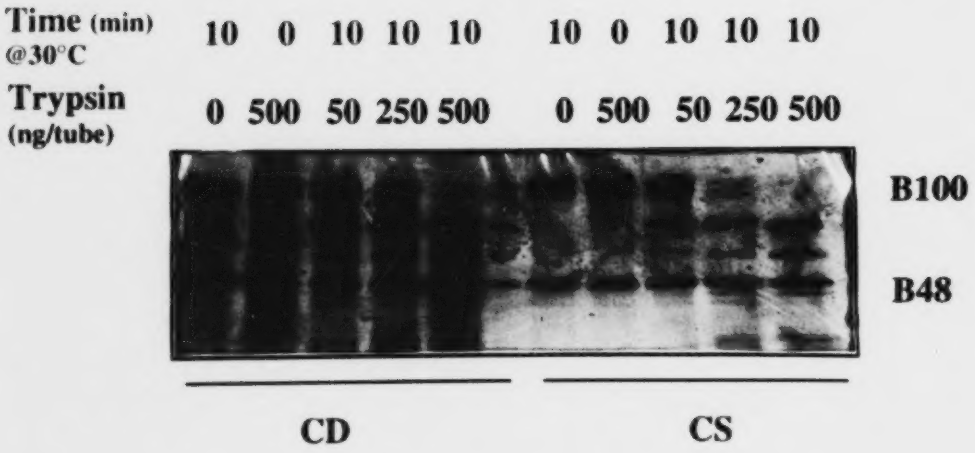
The studies by electron microscopy suggested that there were more large particles in plasma from CD than from CS rats. We investigated if additional differences might be detected in plasma VLDL from CD and CS rats. Several other groups have investigated changes in apoB conformation by limited proteolysis using cathepsin D (Chen *et al.*, 1991), thrombin (Leroy *et al.*, 1992), and trypsin (Cardin *et al.*, 1984). We used a limited proteolysis of plasma VLDL from CD and CS rats to monitor changes in apoB conformation caused by choline deficiency. The apolipoprotein composition of the CS- and CD-derived plasma lipoproteins was similar as judged by staining of proteins on SDS-PAGE gels (Yao and Vance, 1990).

3.9.3.1 Trypsin

The results of treatment of CD and CS plasma VLDL with trypsin are shown in Figure 34. The apoB100 and apoB48 in plasma VLDL from CD rats was more resistant to digestion by trypsin than was that in VLDL from CS rats. The major peptides formed by digestion with trypsin were the same

Figure 34. Tryspin Digestion of ApoB in VLDL from the Plasma of CD and CS Rats.

VLDL protein (20 µg) from either CD or CS plasma was incubated in the presence of 0 to 500 ng trypsin in a buffer that contained 10 mM Tris-HCl (pH 7.4) for 10 min at 30°C. The reaction was stopped by the addition of an equal volume of sample buffer [12.5 mM Tris-HCl, pH 6.8, 4% SDS, 10% (v/v) ß-mercaptoethanol, 20% glycerol, 0.04% bromophenol blue] and immediately boiled for 5 min. The samples were electrophoresed on a 3-15 % polyacrylamide gel that contained 0.1 % sodium dodecyl sulfate, and silver stained.


Time (min) @30°C
10 0 10 10 10 10 0 10 10 10
Trypsin (ng/tube)
0 500 50 250 500 0 500 50 250 500
B100
B48
CD
CS

from the CD and CS rats. We calculated the first order rate constants as the differences in proteolysis of apoB was a kinetic function. From the gels of the incubations, we scanned for the amounts of apoB100 and apoB48 (CAMAG TLC Scanner II, 460 nm) and calculated first order kinetic constants as shown in Table VII. This could not be done for apoB48 from the trypsin digests as it appeared that a breakdown product of the apoB100 migrates with apoB48. It, therefore, seems that not only is the average size of the VLDL particles larger in CD than in CS plasma, but that the apoB may have a different conformation on the surface of some of the VLDL particles that alters its susceptibility to proteases.

3.9.3.2 Cathepsin D

Next, we incubated VLDL from the plasma of both CD and CS animals with cathepsin D as a function of time and amount of protease (Figure 35). Cathepsin D appeared to degrade apoB100 more rapidly in the VLDL isolated from the CD compared to CS rats. This is in contrast to trypsin treatment of VLDL. However, apoB48 was degraded less rapidly in the VLDL isolated from the CD compared to the CS rats. The peptides formed during cathepsin D incubations of plasma VLDL were similar for CD and CS rats. The rate constants for the cathepsin D digestion of apoB100 and apoB48 were determined as described for trypsin (Table VII).

3.9.3.3 Thrombin

We also treated plasma VLDL from CD and CS rats with thrombin as a function of time (Figure 36). ApoB100 from VLDL of CS rats is degraded more rapidly than corresponding CD samples. The rate of apoB48 degradation by thrombin is approximately equivalent between CD and CS VLDL. Even at very high concentrations of thrombin, apoB100 from CD VLDL does not appear to be degraded, suggesting that the thrombin accessible

Figure 35. Cathepsin D Digestion of VLDL from Plasma of CD and CS Rats.

VLDL protein (20 µg) from either CD or CS plasma was incubated in the presence of 0 to 20 µg cathepsin D in a buffer containing 37.5 mM sodium acetate (pH 4.8) for 20 min at 30° C. The reaction was stopped and the samples analyzed as described in the Legend to Figure 34.

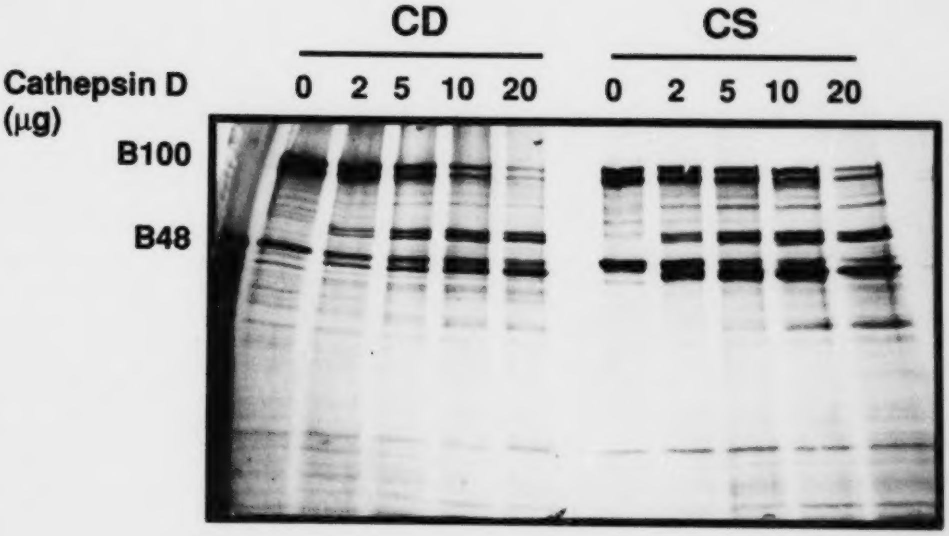

CD
CS
Cathepsin D
(μg)
0
2
5
10
20
0
2
5
10
20
B100
B48

Figure 36. Thrombin Digestion of VLDL from Plasma of CD and CS Rats

VLDL protein (20 µg) from either CD or CS plasma was incubated in the presence of 0-5000 ng thrombin in a buffer containing 50 mM Tris-Cl, pH 8.3 for up to 2 h at 37°C. The reaction was stopped by the addition of an equal volume of sample buffer (12.5 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 10% (v/v) ß-mercaptoethanol, 20% glycerol, 0.04% bromophenol blue) and immediately boiled for 5 min. The samples were electrophoresed on a 3-15% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was incubated with rabbit anti-rat VLDL antibody, as the primary antibody, followed by peroxidase linked goat anti-rabbit IgG. Detection was by enhanced chemiluminescence. The experiment was repeated two times with similar results.

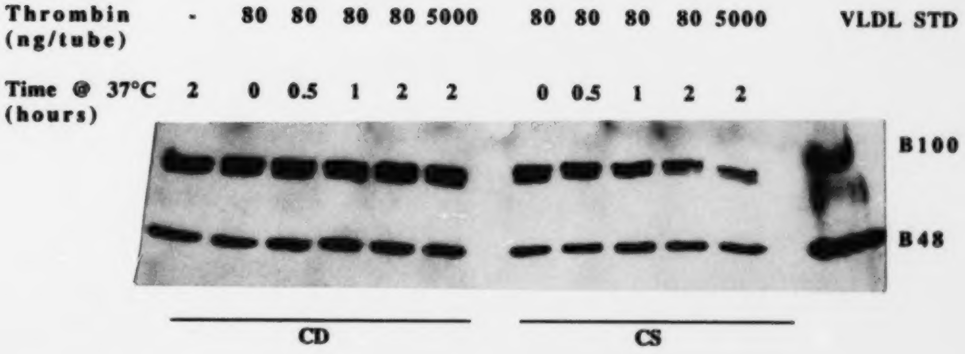

Thrombin (ng/tube)
- 80 80 80 80 5000 80 80 80 80 5000 VLDL STD
Time @ 37°C (hours)
2 0 0.5 1 2 2 0 0.5 1 2 2
B100
B48
CD
CS

Table VII

Rate Constants of VLDL Degradation by Proteases

Plasma VLDL from CD and CS rats was treated with proteases as described in Figures 34 and 36. The bands corresponding to apoB100 or apoB48 were scanned and the k values for first order kinetics of degradation calculated. The results are expressed as densitometry units per µg protease

	apoB100		apoB48	
Protease	CD	CS	CD	CS
Cathepsin D	3.8×10^{-2}	1.1×10^{-2}	1.0×10^{-2}	2.8×10^{-2}
Trypsin	1.73×10^{-3}	3.4×10^{-3}	na	na

na= not analysed due to interference of apoB100 breakdown products sites are not accessible to the protease due to conformational changes in apoB. When human or rabbit LDL was degraded with thrombin, specific bands were produced (Leroy *et al.*, 1992; Cardin *et al.*, 1984). We did not observe any such bands in digestion of either CD or CS VLDL with thrombin. This may be due to a loss of immunoreactivity, or further digestion of apoB fragments. These results show that apoB from CD and CS plasma VLDL have different sensitivities to proteases and are similar to those observed when CD and CS plasma VLDL was subjected to limited proteolysis with cathepsin D and trypsin. Thus, it appears that the apoB conformation or accesibility on plasma VLDL from CD rats is slightly different from that of CS rats. The nature of the conformational changes was not examined.

3.10 Lipid Composition of Subcellular Membranes

We next investigated if the lipid composition of the nascent VLDL particles reflected the lipid composition of the ER or Golgi membranes and if the membranes compensated in some way for impaired PC biosynthesis. We isolated the membranes of ER and Golgi and liver homogenate samples. In all CD samples, there were decreased levels of PC (Table VIII). In liver homogenates, the levels of PE/mg protein did not significantly change, however, the levels of PE increased in both the ER and Golgi membranes from CD livers. The increase in PE is not enough to compensate for the decrease in PC observed in all CD fractions examined. In each case the ratio of PC/PE is lower in CD relative to CS (Figure 37). Thus, it would appear that the membranes of the ER and Golgi compensate for the decreased levels of PC by increasing the amount of PE. The levels of the minor phospholipid components were not changed in CD vs. CS subcellular fractions (Table VIII). The PC/PE ratio of the subcellular fractions from CS rats agrees well with a previous study that has examined the amounts of PC and PE in the ER and

Table VIII

Lipid Composition of Subcellular Membranes Isolated from Rat Liver

Subcellular fractions were isolated from CD and CS rat livers, and the membranes isolated after treatment of ER and Golgi with sodium carbonate. Liver homogenates were also examined. Protein concentration of the membranes was measured and lipids extracted. The masses of PC, PE, phosphatidylinositol and phosphatidylserine were measured. The results are mean nmol/mg protein ± S.E.M. of at least 3 separate experiments.

Fraction	Phospholipid	CD	CS
Liver Homogenate	PC	30.1 ± 3.6[a]	52.6 ± 3.9
	PE	26.3 ± 3.9	25.2 ± 3.9
	PI	3.0 ± 2.5	3.1 ± 2.4
	PS	3.8 ± 2.3	4.0 ± 2.6
ER-membrane	PC	204.7 ± 26.6[a]	277.4 ± 34.3
	PE	146.6 ± 17.8[a]	102.4 ± 7.7
	PI	15.3 ± 6.9	11.6 ± 5.7
	PS	14.5 ± 4.2	12.0 ± 3.2
Golgi-membrane	PC	258.8 ± 43.7[a]	352.8 ± 47.5
	PE	177.1 ± 29.8[a]	117.3 ± 21.1
	PI	16.5 ± 6.4	14.9 ± 7.2
	PS	14.8 ± 4.6	12.9 ± 4.6

[a] $p < 0.05$ CD vs. CS

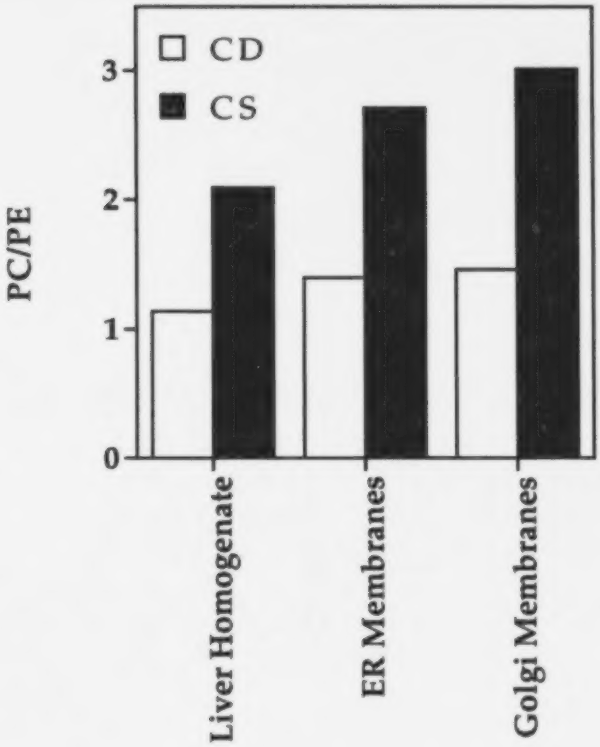


Figure 37. PC/PE Ratio of Subcellular Membranes from Rat Liver

Liver homogenate, ER and Golgi membranes were isolated as described in Table VIII. The ratio of PC/PE in each fraction are shown here.

Golgi membranes (Keenan and Morré, 1970). Our results show that the changes in nascent VLDL phospholipids from CD animals (Table IV) mimicked the organelle phospholipid composition from which they were isolated (Table VIII). Possibly, the membrane composition of secretory organelles is a factor in determination of the phospholipid coat of VLDL particles.

3.11 Protease Inhibitor Studies

Protease inhibitor studies were undertaken to attempt to define the nature of the protease(s) involved in CD specific degradation of apoB. The basic protocol involved labeling the cells with [^{35}S] cell labeling mixture in the presence or absence of various protease inhibitors. After up to 16 hours of labeling, the cells and medium were collected and VLDL immunoprecipitated. Proteins were separated by SDS-PAGE and the gels subjected to fluorography. Cell viability in the presence of protease inhibitors was assayed by Trypan blue dye exclusion, and by release of lactate dehydrogenase into the medium. Unfortunately, the results from the protease inhibitor studies are inconclusive and do not clearly define the type or localization of the protease(s) involved in apoB degradation in choline deficiency.

3.11.1 ALLN

ALLN has been used successfully in the past to block the ER degradation of apoB (Thrift *et al.*, 1992; Sakata *et al.*, 1993 Du *et al.*, 1994; Adeli, 1994). ALLN is a calpain protease inhibitor. ALLN has been shown to block the degradation of other ER based degradations such as HMG-CoA reductase (Inoue *et al.*, 1991). However, it does not block the ER degradation (Stafford and Bonifacino, 1991) of the α chain of the T-cell receptor (Inoue and Simoni, 1992). I attempted to block the intracellular degradation of apoB

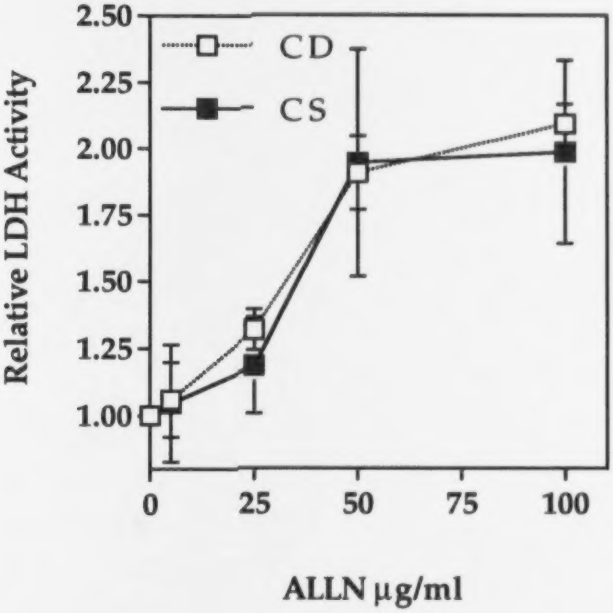


Figure 38. Lactate Dehydrogenase (LDH) Release in the Presence of ALLN

CD or CS hepatocytes were incubated in the absence (0.01% ethanol) or presence of ALLN (0 - 100 µM) for 3 hours. Media were harvested and centrifuged for 10 min at 1000 x *g*. LDH activity was measured using 20 µl of medium. The data are expressed as the release of LDH into the medium relative to the absence of ALLN. The values are means ± S.E.M. of 4 separate experiments.

with ALLN. However, the ALLN was toxic to the primary hepatocytes. The cell death was monitored in two ways: first, a large number of cells did not exclude Trypan blue, even at very low concentrations (5 μM) of ALLN (data not shown); and second, I monitored the medium for lactate dehydrogenase release. The relative amount of lactate dehydrogenase activity in medium from both CD and CS hepatocytes increases dramatically when ALLN is present (Figure 38). Thus I could not determine if ALLN sensitive proteases were involved in apoB degradation in choline deficiency.

3.11.2 Chloroquine and Ammonium Chloride

To determine if the degradation of apoB observed in CD cells were occurring in the lysosomes, two commonly used lysosomal protease inhibitors were added to CD and CS hepatocytes. As shown in Figure 39, the presence of 20 μM chloroquine did not increase the label of apoB48 in either CD or CS cells relative to control values. Due to poor labeling of apoB100, the effect of chloroquine on apoB100 could not be determined. The smaller apolipoproteins (E and A1) were more efficiently labeled than apoB, and this accounts for the greater intensity of the small apoproteins on the autoradiographs. Higher concentrations of chloroquine were toxic to the cells as measured by Trypan blue exclusion (data not shown). However, the addition of chloroquine did prevent the appearance of a pair of bands between apoE and apolipoprotein AI, and caused increased levels of apoAIV intracellularly, suggesting that chloroquine was blocking lysosomal degradation of proteins.

3.11.3 TLCK

To determine if trypsin-like proteases were involved in apoB degradation in CD hepatocytes, TLCK (50 μg/ml) was used to block trypsin-

Figure 39. Effect of Chloroquine and TLCK on Intracellular ApoB

CD and CS hepatocytes were cultured for 16 hours in the presence 15 µCi ^{35}S cell labeling mixture and either 20 µM chloroquine, 50 µg/ml TLCK or no addition (control) After the incubation, cells were harvested and VLDL immunoprecipitated. Proteins were separated by SDS-PAGE after being denatured in 2X SDS-PAGE sample buffer by boiling for for 5 min. Radioactivity associated with proteins was detected by fluorography. The experiment was repeated twice with similar results.

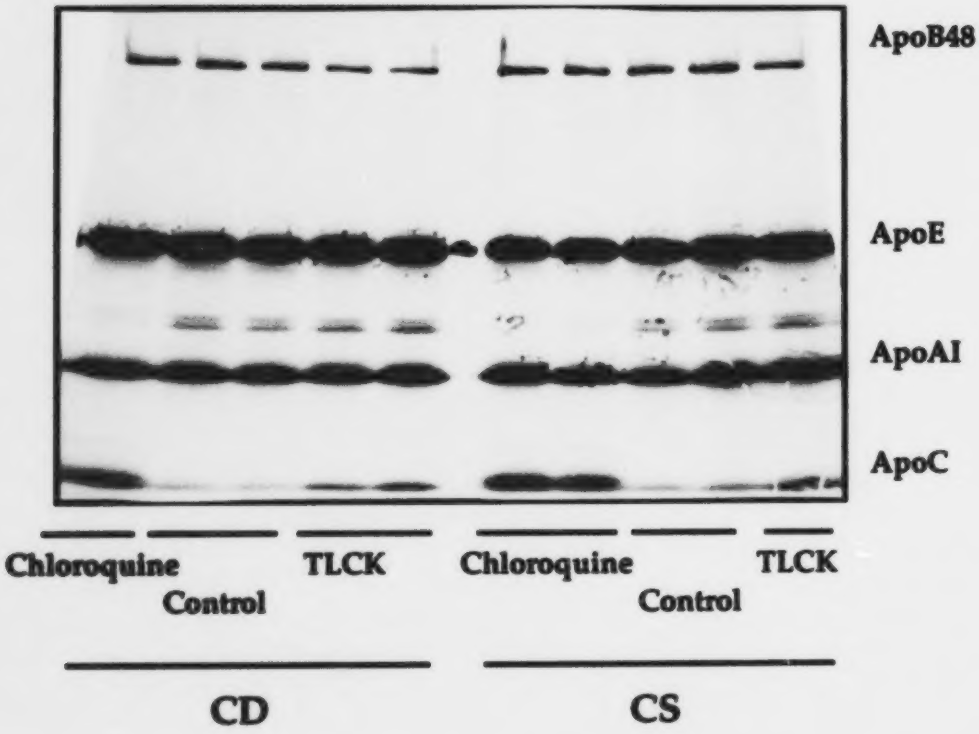
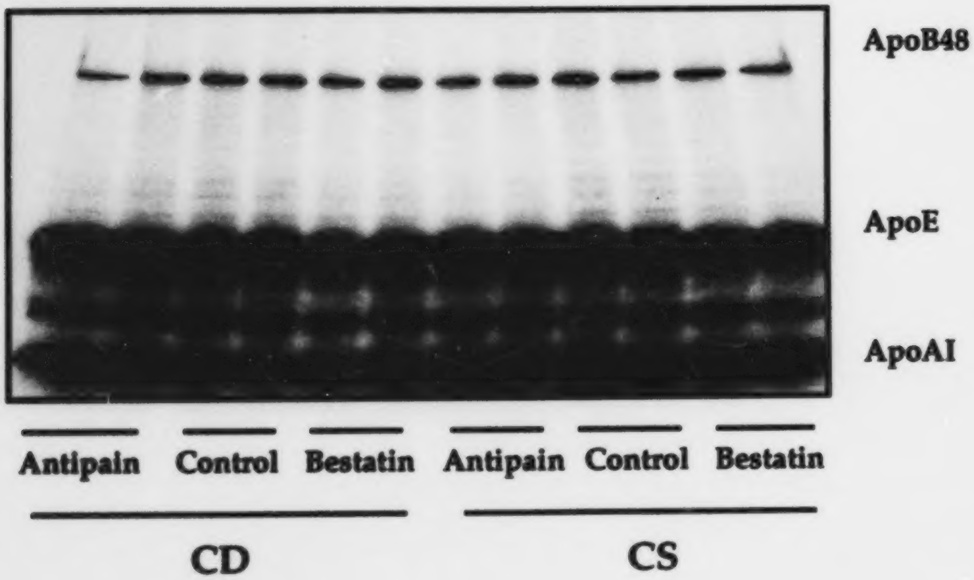

ApoB48
ApoE
ApoAI
ApoC
Chloroquine
Control
TLCK
Chloroquine
Control
TLCK
CD
CS

Figure 40. Effect of Antipain and Bestatin on Intracellular ApoB48

Hepatocytes were cultured as in Figure 39, except either antipain (8 μg/ml) or bestatin (8 μg/ml) were used as protease inhibitors. After the incubation, cells were harvested and VLDL immunoprecipitated. Proteins were separated by SDS-PAGE after being denatured in 2X SDS-PAGE sample buffer by boiling for 5 min. Radioactivity associated with proteins was detected by fluorography. The experiment was repeated twice with similar results.


ApoB48
ApoE
ApoAI
Antipain
Control
Bestatin
Antipain
Control
Bestatin
CD
CS

like activity. However, no protection of intracellular apoB was observed in the presence of TLCK (Figure 39). Suggesting that trypsin was not involved in the degradation of CD apoB48. Due to poor labeling of apoB100, the effect of TLCK on apoB100 could not be determined.

3.11.4 Antipain and Bestatin

Antipain (8 μg/ml), an inhibitor of trypsin and papain type proteases and bestatin (8 μg/ml), an inhibitor of aminopeptidase B, were added to primary hepatocytes from CD and CS cells in an attempt to block the CD specific degradation. However, no protection of intracellular apoB48 was observed in the presence of either antipain or bestatin (Figure 40). ApoB100 was not observed due to poor labeling.

3.11.5 Other Protease Inhibitors

Preliminary experiments with other protease inhibitors (soybean trypsin inhibitor, leupeptin, N-ethylmaleimide and PMSF) did not affect the amount of intracellular, or secreted apoB under similar conditions (data not shown).

4 Discussion

The results from these experiments provide new insights into the mechanism by which impaired PC biosynthesis in CD hepatocytes inhibits the secretion of VLDL. When this study was initiated, the working hypothesis was that decreased PC biosynthesis, as a result of choline deficiency, interfered with an early stage of VLDL assembly. This was thought to be most likely localized to the ER, where PC is added to apoB and nascent VLDL particles are formed. A model of the initial hypothesis of apoB assembly in normal and CD states is shown in Figure 1. The results of this study are discussed below and a new model of the role of PC biosynthesis in VLDL assembly and secretion is proposed.

4.1 Evidence for ER Assembly of Nascent VLDL in Choline Deficiency

Initially, I used subcellular fractionation to determine the site of impaired VLDL assembly and/or secretion. In the normal state, VLDL are assembled in the ER (Rusiñol *et al.*, 1993a). The synthesis of apoB is normal as is shown by equivalent amount of label incorporated into apoB100 and apoB48 in both CD and CS states in the presence of BFA (Section 3.6.1). This confirms previous work using this system (Yao and Vance, 1988). Using subcellular fractionation of whole liver from CD and CS rats, I have shown that the amount of apoB in the ER lumina is equivalent from CD and CS states (Figures 6 - 8, Table III). ApoB levels in ER and Golgi lumina were examined by semi-quantitative immunoblot analysis and by quantitative ELISA. Unfortunately, the ELISA technique cannot distinguish between apoB100 and apoB48. From the density analysis of apoB distribution in ER and Golgi lumen, we can see that nascent VLDL particles are formed in the ER in both CD- and CS-states (Figure 8). ApoB100 is found mainly in the

VLDL density range in ER lumina, but is in both the d< 1.01 g/ml and d≥ 1.01 g/ml fractions in Golgi lumina (Figure 8). In plasma, apoB100 floats in VLDL and IDL ranges (Hussain *et al.*, 1989), and this may account for the distribution of apoB100 in both the d< 1.01 g/ml and d≥ 1.01 g/ml fractions of Golgi lumina (Figure 8). However, the lack of apoB100 in the d≥ 1.01 g/ml fraction of ER lumina cannot be accounted for, and may be explained by the inability to detect the low levels of apoB100 present in ER lumina. ApoB48 is found in both the d< 1.01 g/ml and d≥ 1.01 g/ml fractions of both ER and Golgi lumina. However the relative amount of apoB48 in the d≥ 1.01 g/ml fraction is greater in the ER lumina than in the Golgi lumina for both CD and CS samples. This suggests that a portion of apoB48 is not immediately formed into VLDL sized particles in the ER, and may involve subsequent addition of lipid prior to, or upon arrival in the Golgi (Borén *et al.*, 1994; Alexander *et al.*, 1976). Alternatively, the heavier particles may be selectively degraded prior to arrival in the Golgi. These two possibilities cannot be distinguished from this set of experiments, but the data do not exclude either possibility. ApoB100 does however, appear to be formed into VLDL like particles in the ER lumen suggesting that it is assembled into VLDL in a single step (Rusiñol *et al.*, 1993a; Borén *et al.*, 1994). The relative amounts of apoB48 and apoB100 in the ER lumina of CD and CS rats appear to be equivalent, and there appears to be less apoB48 and apoB100 in the Golgi lumina of CD, than in CS rats (Figure 7 and Table III). Lipid analysis of the material in the d< 1.01 g/ml fraction of both CD and CS ER and Golgi lumina showed that they did contain TG (Table IV).

I also examined the possibility that translocation into the ER lumina was impaired in CD by treating intact microsomes from CD and CS hepatocytes with trypsin (Figure 9). If fewer particles were present in the lumina from CD than from CS microsomes, there should have been less luminal apoB present after trypsin digestion. However, the amount of apoB that remained after trypsin digestion was the same for CD and CS derived samples. This study showed that equal amounts of apoB100 and apoB48 were protected in the lumina of the microsomes from both CD and CS samples (Figure 9). This also confirms previous work that shows a relatively large proportion of apoB is exposed on the cytosolic surface of the ER (Davis *et al.*, 1990; Dixon *et al.*, 1992; Wilkinson *et al.*, 1992b). The membrane bound form of apoB is not thought to be involved in VLDL assembly, but rather is thought to be degraded. The experiments showing that an equivalent portion of apoB is protected in CD and CS states also suggest that a large portion of apoB is not used for lipoprotein assembly in either the CD or CS states. Moreover, *in vitro* transcription-translation studies with carboxy-truncated apoB (apoB15) show no defect in the ability of apoB15 to translocate into the lumen of CD compared to CS microsomes (Vermeulen, P.S., Rusiñol, A., and Vance, D.E., unpublished results). Taken together, the results suggest that translocation of apoB is not a limiting factor in the production of VLDL in choline deficiency. This is in contrast to enrichment of microsomal membranes with phosphatidylmonomethylethanolamine, an analog of PC that lacks two methyl groups in the head group moiety. In phosphatidylmonomethylethanolamine enriched microsomes, translocation of apoB into the lumen is impaired (Rusiñol *et al.*, 1993b), and this blocks the secretion of VLDL (Vance, 1991). However, bulk lipid changes do not appear to affect protein translocation and are not thought to be directly involved in protein translocation (Simon and Blobel, 1991). However, the altered phospholipids may indirectly affect the conformation of the protein translocation channel, or prevent correct assembly of lipid with apoB, and thus affect translocation of apoB in the phosphatidylmonomethylethanolamine enriched microsomes.

4.2 Lipoproteins Formed in Choline Deficiency Have an Altered Structure

Since the initial hypothesis that VLDL particle formation was impaired in the ER lumina of CD liver was incorrect, I characterized the particles in the lumina of the secretory pathway, as well as the particles from plasma of CD and CS rats. The secretion of particles from CD livers appears to be impaired after the ER. The amount of apoB is decreased in Golgi lumina from CD relative to CS liver and no accumulation of apoB100 or apoB48 was observed in the ER lumina of CD livers (Figure 7 and Table III). Hence, the decrease in plasma VLDL observed in choline deficiency was not due to impaired or delayed secretion.

One possibility to explain decreased VLDL secretion is that the particles formed in choline deficiency are defective and are recognized by a quality control mechanism that would prevent particles generated in CD conditions from being secreted. Therefore, I analyzed the lipid composition of VLDL from ER and Golgi lumina VLDL and from plasma. The PC/PE ratios of CD and CS d< 1.01 g/ml fractions from ER lumina were not significantly different (Figure 29). However, the ratio of PC/PE in the d< 1.01 g/ml fraction of Golgi lumina from CD is lower than that of CS rats (Figure 29). I also measured the relative amounts of PC, PE and TG per µg apoB in the lumina contents (Table V). From this data it is apparent that the CS particles from both ER and Golgi lumina are enriched in PC per µg apoB relative to particles from CD samples. The particles in the Golgi but not the ER lumina from CD liver were enriched in PE per µg apoB relative to particles from CS rat liver, suggesting that the lipid composition of the particles is altered in choline deficiency. The amount of TG per apoB was approximately equal in

CD and CS samples from ER lumina, but was lower in CD compared to CS Golgi lumina (Table V). However, the calculations do not account for the distribution of apoB in both the d<1.01 g/ml and the d≥ 1.01 g/ml fractions, nor do they account for differences in the amount of lipid associated with apoB100 and apoB48. To determine the relative size and extent of lipid loading of the particles in the lumina of ER and Golgi, as well as from plasma of CD and CS rats, I examined the ratio of TG to the major phospholipids (Figure 30). It is not likely that the VLDL particles formed in CD hepatocytes are defective in TG loading by microsomal lipid transfer protein (Gordon *et al.*, 1994; Leiper *et al.*, 1994) as VLDL particles isolated from the ER and Golgi lumina (Figure 30) and plasma (Figure 32) have similar or higher ratios of TG/(PC + PE) for CD compared to CS, derived samples. The TG/(PC + PE) ratio does not accurately reflect particle diameter as it does not take into account, minor phospholipids or cholesterol associated with lipoprotein particles. For this reason, a calculation of core volumes and particle diameters was not performed with this data. Although, the data generally support the electron microscopy observations that there are larger particles present in the VLDL isolated from CD Golgi lumen and plasma than from corresponding CS fractions. However, it is noteworthy that the ratio of TG/(PC + PE) in the ER is approximately half that found in the Golgi for both CD and CS samples (Figure 30). This suggests that there may be a subsequent addition of lipid to nascent VLDL particles that takes place in a post-ER compartment (Borén *et al.*, 1994, Alexander *et al.*, 1976). However, it does not explain the observation that apoB100 and apoB48 can be found in the VLDL density range of ER lumina. In CD livers there is an accumulation of cytosolic TG (Yao and Vance, 1988; 1990). Since TG assembly into VLDL is apparently normal, accumulation of

TG in choline deficiency is apparently due to excess synthesis, probably utilizing diacylglycerol, which in CS hepatocytes would be used for PC biosynthesis. In addition, perhaps this excess TG is derived from the VLDL particles expunged from the secretory system in the CD hepatocytes. The CD VLDL from both Golgi lumina and plasma appear to be slightly larger than the corresponding CS samples when analyzed by negative staining electron microscopy (Figures 28 and 31). This is interesting since the plasma TG is much lower in CD than in CS rats (Yao and Vance, 1990). A similar phenomenon, of larger particle size in the presence of lower plasma TG, has been observed in human studies where LDL size has been shown to be inversely related to plasma TG concentration (McNamara *et al.*, 1992). Although, this is caused by a different mechanism than a decrease in the amount of PC biosynthesis.

The increase in the amount of TG per particle observed in choline deficiency, is similar to that observed in patients with Tangier's disease. These patients have LDL that is enriched in TG (Kunitake *et al.*, 1990). When LDL from Tangier's patients is subjected to limited proteolysis, changes in the susceptibility of apoB to degradation have been observed (Kunitake *et al.*, 1990). A similar change in protease sensitivity of apoB has been observed when LDL and VLDL from human plasma were treated to limited proteolysis. The apoB on larger particles (VLDL) showed a different sensitivity to proteases than did apoB on smaller particles (LDL), suggesting that the conformation of apoB on VLDL was different from that of LDL (Chen *et al.*, 1991). Since the TG/(PC + PE) ratios and EM studies of plasma VLDL suggested that particles from CD samples are larger than corresponding CS samples, we used limited proteolysis to examine apoB from CD and CS plasma VLDL for conformational differences. From the treatments with trypsin, cathepsin D, and thrombin (Figures 34 - 36), we showed that both apoB100 and apoB48 did have different protease susceptibilities in CD compared to CS samples. Further evidence that abnormal particles are produced when PC biosynthesis is inhibited came from electron microscopy measurements of VLDL diameter. Particles from both Golgi lumina and plasma of CD rats were larger than corresponding CS fractions (Figures 28 and 31). Thus the conformational differences in apoB observed in CD vs. CS plasma VLDL may be due to altered phospholipid composition, changes in size of the particle, or a combination of these two factors. The implications of the altered apoB conformation on CD VLDL compared to CS VLDL is unknown at this time.

In both CD and CS samples there is a relative enrichment of the PC/PE ratio in plasma VLDL compared to nascent Golgi luminal VLDL (CD; 6.4 vs. 1.13, CS; 12.6 vs. 3.50). This is consistent with the findings of Hamilton and Fielding (1989) who showed that nascent Golgi VLDL were enriched in PE relative to plasma VLDL. Presumably this is due to net exchange of PE to other lipoproteins such as HDL in the plasma, or selective lipolysis. HDL levels in plasma are not affected by choline deficiency (Yao and Vance, 1990). Lipid analysis of the HDL fraction of CD and CS plasma showed no significant differences in either the PC/PE ratio (Figure 33), or the TG/(PE + PE) ratio (Figure 32). This data suggests that PC levels are maintained in CD HDL. The lipid analysis confirms the earlier result that HDL is not affected by decreased PC biosynthesis (Yao and Vance, 1988).

These observations suggest that impaired PC biosynthesis, resulting from choline deficiency, does not limit formation of nascent VLDL in the ER lumina, but rather suggest that particles with altered size and phospholipid composition are generated.

4.3 Altered Particles Produced in Choline Deficiency are Recognized and Degraded by a Quality Control Protease in a Post-ER Compartment

As discussed above nascent VLDL are synthesized at the same rate (Yao and Vance, 1988) and found in equal concentrations in ER lumina of CD and CS rats, and these particles have an altered lipid composition relative to CS particles. Therefore, the original hypothesis that assembly of VLDL would be impaired in choline deficiency is incorrect, and an alternate mechanism must be proposed. There are two potential models; 1. nascent VLDL particles in CD are transported through the secretory pathway at a decreased rate relative to CS particles, or 2. particles produced in choline deficiency are preferentially degraded. If the transport of nascent VLDL was inhibited in CD, presumably between the ER and Golgi to account for decreased apoB in Golgi lumina, we should observe an accumulation of apoB in the lumina of CD ER. However, the levels of apoB100 and apoB48 were not significantly increased in CD relative to CS ER lumina. Therefore, the first model must be incorrect. We then set out to test the second model, and to determine the site of degradation of CD particles.

We used BFA to disrupt the transport of proteins from the ER to the Golgi. Moreover, the Golgi tends to redistribute into the ER (Klausner *et al.*, 1992; Lippincott-Schwartz, 1993), and therefore experiments containing BFA and nocodazole were performed to ensure that retrograde transport of Golgi to ER was not interfering with the interpretation of the results. If the degradation of VLDL from CD livers were occurring in the ER, BFA treatment should result in degradation of apoB in the ER. Consequently, less apoB would be present in CD hepatocytes when compared to similarly treated CS hepatocytes. However, if the CD specific degradation was in a post-ER compartment, BFA treatment should result in protection of apoB and the amount of apoB in CD hepatocytes would be equivalent to that found in CS hepatocytes also treated with BFA. Treatment of cells with BFA does not accelerate apoB degradation in the CD compared to CS hepatocytes (Figures 11 and 13). Treatment of the cells with monensin, an inhibitor of transport through the *trans*-Golgi did not conclusively define the site of degradation, but do not contradict the studies where transport of proteins was blocked with BFA or BFA plus nocodazole.

To account for these findings, we postulate the existence of a quality control protease in a post-ER compartment that recognizes the apoB in the abnormal particles and initiates apoB degradation. This proposal has one limitation since particles with a larger size and abnormal phospholipid composition are observed in plasma from CD rats (Figures 31 - 33). It is also possible that in the presence of BFA an ER based protease would be overwhelmed with higher affinity substrates and therefore not efficiently degrade apoB. This would imply that if there is a quality control protease, it is not 100% efficient. The exact location of apoB degradation and the disposition of the lipid core are presently unknown, it is possible that apoB is cleaved , but still remains associated with the nascent VLDL particle. This altered particle may then be targeted to lysosomes or peroxisomes for degradation of apoB and disposition of the lipid core. Ubiquitination of apoB has not been examined and this may also be a potential mechanism for targeting apoB from CD cells to a degradative pathway. It is also not clear why the hepatocytes would bother to degrade these abnormal particles rather than secrete them. The signal for degradation may be simply that the particles with abnormal phospholipid coats are unstable, spontaneously aggregate and precipitate, in a manner similar to treatment of LDL with phospholipase-C (Liu *et al.*, 1993). The precipitated apoB could then be targeted to any number of proteases and apoB fragments may not be detected by conventional methods due to a loss of immunoreactivity. It is also possible, that nascent VLDL size may be a signal for degradation. This hypothesis is supported by the work of Wang *et al* .(1994) who showed using carboxy-terminal truncated apoBs expressed in McA-RH7777 cells, that lipid-rich particles generated in the presence of exogenous n-3 fatty acids were preferentially degraded, relative to lipid-poor particles generated under the same conditions. An alternate explanation for the decreased levels of VLDL observed in medium or plasma of CD samples is that re-uptake of newly secreted apoB is occurring in CD liver. This might be caused by an unstirred water layer around the cells as suggested by Williams *et al.* (1990). However, the re-uptake of newly secreted apoB does not appear to be a factor in secretion under normal conditions (Hara *et al.*, 1993). Re-uptake of apoB is not likely a determining factor in the decreased levels of apoB observed in CD compared to CS plasma as the amount of apoB is decreased in late stages of the secretory pathway of CD compared to CS liver (Figure 7 and Table III). However, it is possible that CD and CS VLDL particles have different affinities for lipases or receptors.

Other examples of post-ER degradation include the secretory form of IgM. This protein is synthesized in excess and a significant amount is degraded intracellularly in a post-ER, pre-*trans*-Golgi compartment (Amitay *et al.*, 1991). It is postulated that degradation is a mechanism for regulation of secretion of IgM. An analogous process may be occurring in choline deficiency, where nascent VLDL particles are screened and abnormal particles subsequently degraded

4.3.1 Nature of Protease Involved in CD Specific Degradation of VLDL

We attempted to identify the type of protease activity involved in the CD-specific degradation. However, despite numerous experiments we were unable to find a protease inhibitor (chloroquine, soybean trypsin inhibitor, NH_4Cl, leupeptin, PMSF, TLCK, antipain, bestatin and *N*-ethylmaleimide were tried) which blocked the CD-specific degradation of VLDL. Although, it is possible that not all of the protease inhibitors were efficiently taken up in the cells and did actually inhibit the desired proteases. Therefore, these studies suggest that the CD specific degradation of apoB is not occurring in the lysosomes since degradation was not blocked by chloroquine. ALLN, which has been shown to block the ER degradation of apoB in a variety of cell types (Thrift *et al.*, 1992; Sakata *et al.*, 1993; Adeli *et al.*, 1994; Du *et al.*, 1994), was toxic to rat hepatocytes (Figure 38) and, therefore, could not be used. An attractive candidate for the protease is the multicatalytic proteinase complex (Rivett, 1993; Rechsteiner *et al.*, 1993; Peters, 1994), this is an abundant cytoplasmic protein (1% of soluble cell protein) composed of up to 25 subunits with multiple proteolytic activities. Another candidate, although unlikely due to its subcellular location, is the ER-60 protease, which is localized to the ER and is inhibited by acidic phospholipids (Urade and Kito, 1992). It is possible that the altered phospholipid composition of nascent CD VLDL could make it a target for this protease. Specific degradation of apoB in choline deficiency may also be regulated by the redox potential of the ER. Young *et al.*, (1993) have shown that introducing reducing conditions to the ER enhanced degradation of the CD3 γ subunit and a control protein. However, the effect of decreased PC biosynthesis on the ER redox potential is unknown and it is not likely to be a significant factor concerning this work, since the degradation of apoB observed in choline deficiency is a post-ER process. Other factors that may be responsible for targeting apoB of CD hepatocytes to degradation may include small changes in protein conformation such as an exposed thiol group. This has been shown to be important for targeted degradation of IgM (Fra *et al.*, 1993). An alternative mechanism for the degradation of apoB in CD cells may be the aggregation and subsequent precipitation of unstable particles in a process analogous to treatment of LDL with phospholipase C (Liu *et al.*, 1993).

A further possibility for the CD specific degradation of apoB is that if the rate of secretion of VLDL were decreased as a result of choline deficiency and transport out of ER is impaired, a protease in the ER might degrade apoB, thus causing equivalent levels of apoB in the ER lumina, but decreased levels of apoB in the Golgi lumina of CD, relative to CS liver. If this hypothesis were correct, the putative protease may be localized to the intermediate compartment (Lippincott-Schwartz, 1993) or in the ER itself. However, the experiment in which transport from the ER was blocked with BFA did not show more apoB remaining in CD compared to CS cells. Hence, ER degradation of apoB is unlikely to be responsible for the disappearance of apoB in CD Golgi. Although, this possibility has not been completely eliminated. The nature of the protease activity responsible for the degradation of apoB in choline deficiency needs to be examined further.

4.4 Choline Deficiency and Rate of Protein Secretion

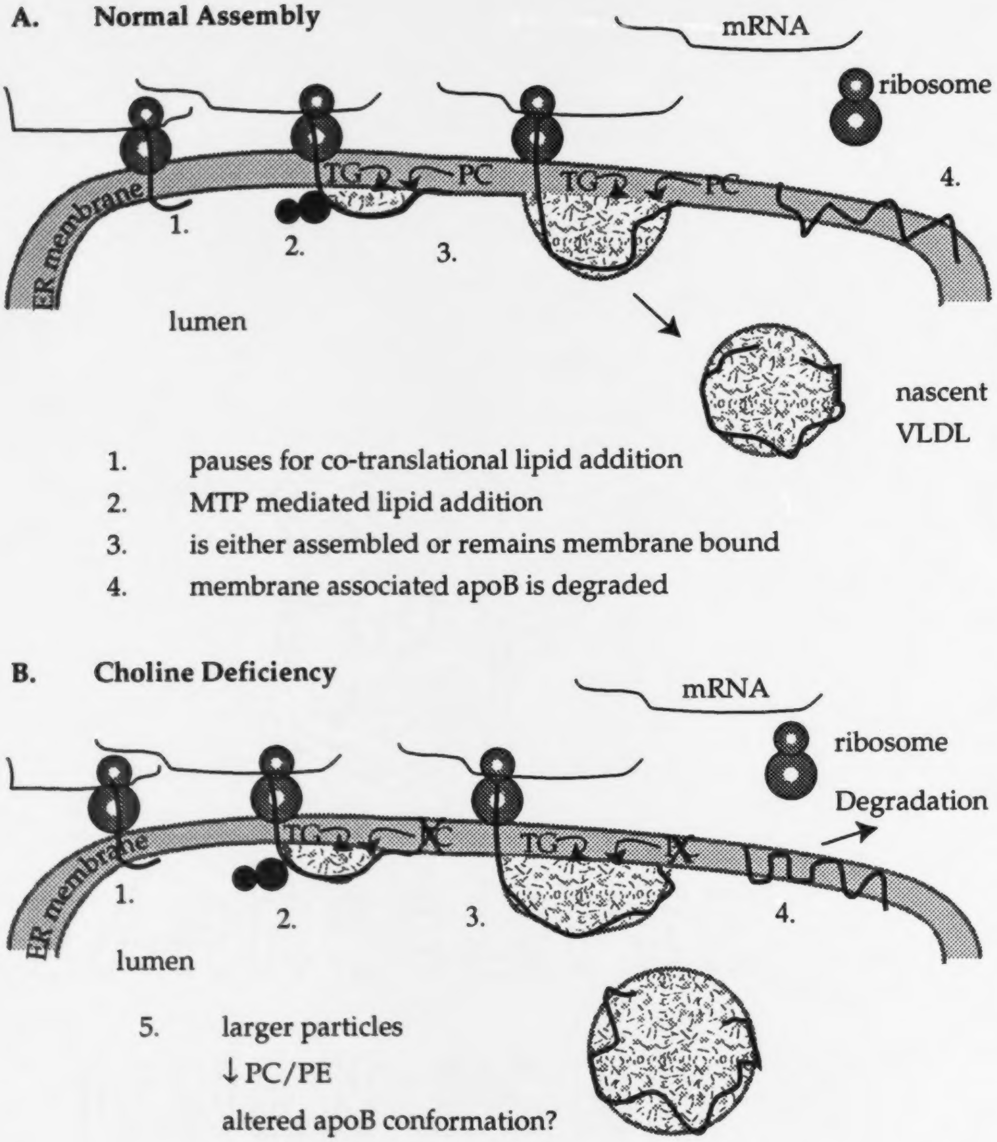
Choline deficiency appears to decrease the rate of bulk protein secretion. The rate of bulk protein secretion in CD samples is decreased to 80% of the CS value (Figure 25). The value for albumin is 60% (at 90 min chase). However, albumin may not be an ideal control protein as it does bind to fatty acids and is involved in lipid metabolism (Spector, 1986). An inhibition of albumin secretion from CD hepatocytes was unexpected, since, in plasma the levels of albumin per mg protein in ER and Golgi lumina and in plasma are equivalent in CD compared to CS rats (Table III). Neither the decreased bulk protein nor albumin secretion is as dramatic as the decrease in apoB secretion under these conditions (40% of the CS value). Hence, while there is a systemic effect on protein secretion, VLDL secretion is more severely affected by choline deficiency. The modest decrease in rate of bulk protein secretion was not previously observed (Yao and Vance, 1988), as protein release into the medium after continuous labeling experiments (12 h) was equivalent from CD and CS hepatocytes. This suggests that choline deficiency delays bulk protein secretion slightly, or is a transient effect that corrects itself at long time points. The alterations in the phospholipid composition of the membrane are likely to affect bulk protein secretion by altering the structure of the membranes, however, the effect on protein secretion caused by the decreased PC and relative increase in PE observed in the ER and Golgi membranes is more pronounced for albumin and VLDL compared to TCA precipitable proteins. This suggests that the mechanism by which VLDL, and possibly albumin, are transported intracellularly may be separate from bulk protein secretion. Therefore, inhibiting the biosynthesis of PC may lead to some interesting insights into intracellular trafficking.

4.5 Relevance of Subcellular Membrane Composition to VLDL Phospholipid composition

The increase in ER and Golgi membrane PE under CD conditions (Table VIII) suggests that the membrane lipid composition is changed to remain functional in the presence of decreased levels of PC. It is also known that under CD conditions, the fatty acid composition of microsomal and nuclear membranes change (Kapoor *et al.*, 1992) with a trend toward longer side chains (arachidonic acid) in microsomes. Changes in fatty acid composition of membrane lipids as a result of choline deficiency was not examined in this thesis, but it is likely that the fatty acid composition of both the membranes and the VLDL produced in choline deficiency are altered relative to the control state. It is also possible that the membrane composition of secretory organelles is a factor in the determination of VLDL phospholipids as the nascent VLDL phospholipid composition mimicked the organelle phospholipid composition from which they were isolated. The fatty acid side chains of phospholipids from nascent VLDL have not been examined to determine if a similar change is occurring in these phospholipids under CD. Thus it appears that VLDL phospholipid is affected by the organelles through which it moves along the secretory pathway.

4.6 Model for Phosphatidylcholine Biosynthesis in VLDL Assembly (1994)

As a result of the studies discussed above, the model of lipoprotein secretion in choline deficiency proposed in Figure 1 must be altered. Therefore, a new model was generated (Figure 41) to incorporate these findings. The major finding of this thesis is that formation of VLDL particles does occur when PC biosynthesis was inhibited. Nascent VLDL particles are formed in the ER lumen of CD rats albeit with altered phospholipid composition, i.e., less PC and more PE. Nascent VLDL from both CD and CS states appear to have similar amounts of TG associated with them, with some evidence pointing to slightly larger particles produced in CD compared to CS samples. The apoB100 particles appear to be formed in a single step into VLDL density particles in the ER lumina. However, it is possible that apoB48 containing particles require a subsequent addition of lipid, presumably in the Golgi. Therefore, in choline deficiency nascent VLDL are generated in equal



A. Normal Assembly

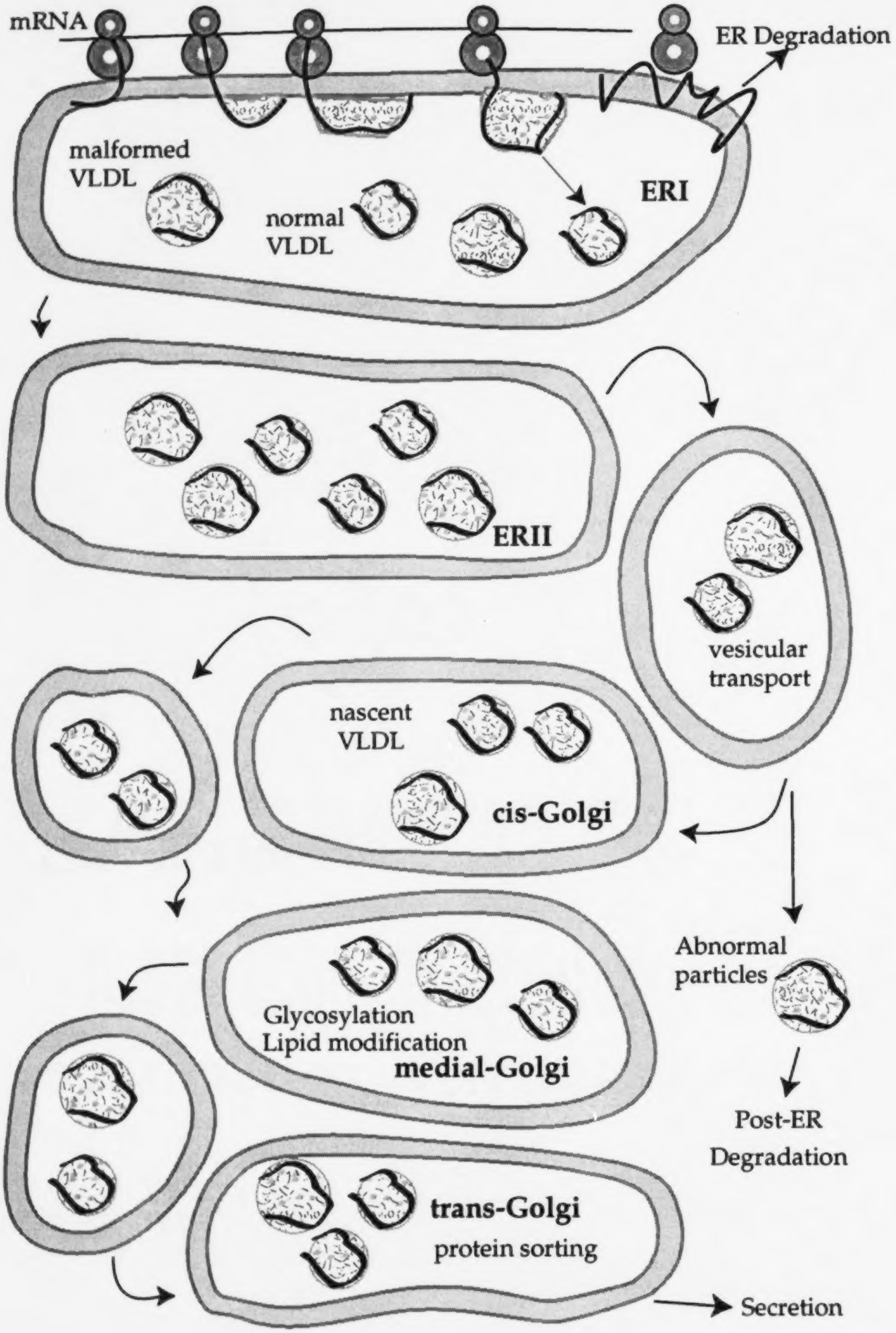
1. pauses for co-translational lipid addition
2. MTP mediated lipid addition
3. is either assembled or remains membrane bound
4. membrane associated apoB is degraded

B. Choline Deficiency

5. larger particles
↓PC/PE
altered apoB conformation?

Figure 41. Models of Lipoprotein Assembly (1994).
The models of lipoprotein assembly in normal (A) or choline deficient (B) states. Particles assembled in CD states are larger, have decreased ratios of PC/PE, and have an altered apoB conformation. These features are thought to target the nascent CD VLDL to a degradation pathway in a post-ER compartment.

Figure 42. Model of Lipoprotein Secretion in Choline Deficient Cells
Lipoproteins are assembled as shown in Figure 41, the larger particles produced in choline deficiency are assembled in the ER lumina and are targeted to a post-ER degradation pathway. Particles are believed to move through the secretory pathway via vesicular transport. Some of the nascent VLDL particles produced in choline deficiency are secreted.


mRNA
ER Degradation
malformed
VLDL
normal
VLDL
ERI
ERII
vesicular
transport
nascent
VLDL
cis-Golgi
Abnormal
particles
Glycosylation
Lipid modification
medial-Golgi
Post-ER
Degradation
trans-Golgi
protein sorting
Secretion

number relative to the CS state, but have an altered phospholipid composition.

4.7 Current Model of VLDL Secretion as Affected by Choline Deficiency

From these studies, a new model of the involvement of PC biosynthesis in VLDL secretion must be drawn (Figure 42). In choline deficiency, impaired PC biosynthesis does not block VLDL assembly in the ER, but larger particles with an abnormal phospholipid coat are generated (Figure 41). The particles formed in choline deficiency do float in the VLDL density range, and do have similar TG/(PC + PE) ratios compared to particles from CS livers. However, there appears to be a selective degradation of particles in a post-ER compartment. This is likely due to either the size of the particles, the phospholipid composition, or the apoB conformation on the particle, although the exact mechanism for the recognition of the defective particles has not been characterized. The nature of the proteolysis occurring in this post-ER compartment has not been clearly defined by this study. The pathway for VLDL secretion, with the targeting of abnormal particles to post-ER degradation, is outlined in Figure 42. However, this model does not account for the presence of VLDL particles with altered size and phospholipid composition in the plasma of CD compared to CS rats.

4.8 Conclusions and Future Considerations

From this work, we can now clarify the role of PC biosynthesis in VLDL secretion. In choline deficiency, nascent VLDLs are formed in the ER with an abnormal phospholipid coat, resembling the membranes from which they are isolated. The decreased PC and increased PE content of the membranes are thought to affect slightly the rate of bulk protein secretion from CD cells, but this effect is not sufficient to account for the decreased

VLDL secretion. The altered phospholipid content of nascent CD VLDL may be involved in targeting these particles to a post-ER proteolytic pathway that is undefined. The targeting of these particles to the degradation pathway is inefficient as the particles that are secreted are abnormal. Future work into the mechanism of impaired PC biosynthesis and VLDL secretion should aim to further define the proteolytic activity responsible for the degradation of apoB in the CD state and also the nature of apoB post-translational modifications that may be resposible for targeting the protein for degradation such as changes in glycosylation or subcellular distribution. Also, the subcellular localization of the degradation observed in choline deficiency should be examined, possibly by utilizing other transport inhibitors or a temperature block. This thesis has not examined the importance of PC biosynthesis in the Golgi and role this may play in the assembly of VLDL, nor has the importance of minor phospholipids such as sphingomyelin been examined. The importance of the fatty acid side chain composition should also be examined. Alternatively, the conformation of apoB may be changed so that a protease sensitive domain is exposed in choline deficiency thus making the nascent particle a target for proteases.

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